A functional comparison of Campath-IH antibodies expressed in and isolated from different cellular sources

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TITLE:

A FUNCTIONAL COMPARISON OF CAMPATH-1H ANTIBODIES EXPRESSED IN AND ISOLATED FROM DIFFERENT CELLULAR SOURCES

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Appendix

THESIS ABSTRACT

Campath-IH is a humanised antibody which has entered clinical trials for both Rheumatoid arthritis and lymphoma. The clinical trial material was expressed in and isolated from Chinese Hamster Ovary (CHO) cells. It is proposed that variations observed in preliminary antibody-dependent cellular-cytotoxicity (ADCC) assays, in which human mononuclear cells were used as effectors and CHO Campath-1H was compared with Campath-1H prepared from rat YO cells, are correlated to host and/or culture condition related changes in antibody N-linked glycosylation.

To investigate the hypothesis, CHO Campath-1H mediated assays including ADCC, monocyte mediated cytostasis, antigen engagement and subsequent crosslinking were analysed as was the antibody N-linked carbohydrate composition. The total removal of the carbohydrate ablated ADCC activity, decreased the cytostatic effect seen with intact antibody but did not alter antigen binding. Campath-1H cDNA was recloned into Celltech Glutamine Synthetase expression vectors and transfected into mouse NSO cells for antibody isolation. Comparisons of antibody made during NSO Campath-1H development from clone to fermentor and between CHO and YO Campath-1H with the various stages of NSO antibody revealed variations in both assay response and N-linked carbohydrate structure. The glycosylphophatidylinositol (GPI)-anchored antigen CDw52 recognised by Campath-1H was isolated from Wien 133 B cell cDNA by polymerase chain reaction and sequenced in preparation for expression cloning. Two sequence variants of the antigen were present in the cDNA, differing by two amino acids outside of the antigen coding region but, at a site controlling GPI-anchor attachment. Both cDNAs were expressed in CHO cell lines for comparison but only one could be detected by Campath-1H. In vivo and in vitro studies on the two forms are described. Chimeric forms of the T cell antigen CD4, linked to either of the CDw52 antigen GPI attachment sequences, were shown to be both expressed in CHO cells and detected by anti-CD4 antibodies.

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DECLARATION:

I declare that, unless stated, I completed all of the experimentation required for this thesis myself.

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This thesis has taken a long time to finish because although the practical work was complete, I lost my way with the writing. Without the support and coercion of my partner Doog, it would probably never have been finished, so I really want to thank him for his persistence.

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ABBREVIATIONS

A	adenosine
a.a	amino acid
Ab	antibody
ADCC	antibody-dependent cell-mediated cytotoxicity
Ag	antigen
ALG	anti-lymphocyte globulin
amp	ampere
ATP	adenosine tri-phosphate
bp	base pair
BSA	bovine serum albumin
C	cytidine or constant region (depending on context)
C'	complement
C1q/C3a/C3b	complement components
^{14}C	carbon nuclide 14
$CaCl_2$	calcium chloride
CD	cluster of differentiation
CDR	complementarity determining region
cDNA	complementary DNA
CDw52	Campath antigen
CHO	Chinese Hamster ovary cell line
CHO C1H	Campath-1H isolated from CHO cells
C 1H	Campath-1H
Con A	concanavalin A
cpm	counts per minute
CTP	cytidine tri-phosphate
CR	complement receptor
d	deoxy
DAF	decay accelerating factor
dd	di-deoxy
DEPC	di-ethyl pyrocarbonate
DHB	dihydroxyl benzoic acid
DMEM	Dulbecco's modified minimal essential medium
DMSO	dimethyl sulphoxide
DNA	deoxy ribonucleic acid
dNTP	nucleoside tri-phosphates
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immuno-sorbent assay
ER	endoplasmic reticulum

Eth	ethanolamine
Fab/F(ab')2	antibody binding fraction of antibody molecule
FACS	fluorescence-activated cell sort or scan
Fc	crystallizable fraction of antibody molecule
Fc R	Fc receptor
FCS	foetal calf serum
Fig.	figure
FITC	fluorescein isothiocyanate
FPLC	fast protein liquid chromatography
Fuc	fucose
G	guanine
Gal	galactose
GalNAc	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
GM-CSF	granulocyte and macrophage colony stimulating factor
GPI	glycosylphosphatidylinositol
GTP	guanosine tri-phophate
h	hour/s
H	heavy chain
^{3}H	tritium
HCl	hydrochloric acid
H_2O	water
HPAEC	high performance anion-exchange chromatography
HPLC	high pressure liquid chromatography
HRP	horse radish peroxidase
H_2SO_4	sulphuric acid
Ig	immunoglobulin
IFN	interferon
IL	interleukin
IMDM	Iscove's modified Dulbecco's medium
IU	international unit
kb	kilobase
K	thousand
L	light chain
LDMS	laser desorption-mass spectrophotometry
LPS	lipopolysaccharide
M	molar
Mab	monoclonal antibody
Man	mannose
MCP	membrane cofactor protein

MACIF	membrane attack complex inhibitory factor
mg	milligram
mg	microgram
$MgCl_2$	magnesium chloride
MHC	major histocompatibility complex
min	minute/s
ml	millilitre
ml	microlitre
mM	millimolar
mM	micromolar
MFI	mean fluorescence intensity
MSX	methionine sulphoximine
MX	methotrexate
MW	molecular weight
NaCl	sodium chloride
NaN_3	sodium azide
NaOH	sodium hydroxide
NaOAc	sodium acetate
Neu	sialyic acid
NK	natural killer
NKSF	natural killer cell stimulatory factor
nm	nanometer
NSO	mouse myeloma cell line
NSO.CF/PF/S	Campath-1H isolated from transfected NSO cells cultured in cholesterol-free, protein-free or serum containing medium respectively
NSO.F	NSO Campath-1H produced in protein-free medium under fermentor conditions
^0C	temperature in degree centigrade
OD	optical density
ORF	open reading frame
%	percentage
^{32}P	phosphorus nuclide 32
PAGE	polyacrylamide gel electrophoresis
PBL	peripheral blood lymphocyte
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PBT	PBS plus BSA and tween
PCR	polymerase chain reaction
PE	phycoerythrin
pFc'	reduced Fc resulting from pepsin cleavage of antibody

PHA	phytohaemagglutinin
PI	phosphatidylinositol
PIG	phosphatidylinositol glycotransferase
PI-PLC	phosphatidylinositol-specific phospholipase C
PMA	phorbol myristate acetate
PMSF	phenylmethylsulphonyl fluoride
PNH	paroxysmal nocturnal haemoglobinuria
PPMN	peripheral blood polymorphonuclear cells
R	receptor
RI	IgG Fc receptor one or CD64
RII	IgG Fc receptor two or CD32
RIII	IgG Fc receptor three or CD16
RA	rheumatoid arthritis
RES	reticulo-endothelial system
RNA	Ribonucleic acid
rpm	revolutions per minute
RT	room temperature
^{35}S	sulphur nuclide 35
sc-Fv	single chain variable fragment
SD	standard deviation
SDS	sodium dodecyl sulphate
SE	standard error
sec	second/s
SPR	specific production rate
SV	Simian virus
T	thymidine
TCR	T cell receptor
TE	Tris/EDTA buffer
TEMED	tetramethylethylenediamine
TGF	transforming growth factor
TNF	tumour necrosis factor
TTP	thymidine tri-phosphate
u or U	unit
UDP	uridine diphosphate
UV	ultra violet
V	variable region
v/v, v/w	volume per volume, volume per weight respectively
YO	rat myeloma cell line
YO C1H	Campath-1H isolated from transfected YO cells

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DEDICATION

I should like to dedicate this thesis to my son Sam, my mum and dad, and also to Doog without whose help and encouragement, it would not have been completed.

CHAPTER ONE: INTRODUCTION

1.1 Aims

The work described in this thesis was designed to investigate the influence of N-linked glycosylation on Campath-1H antibody in defined assays (resulting from antibody expression in different cellular hosts) and antibody/antigen interactions. This introduction reviews topics which may be of importance in the understanding of the data presented.

1.2 Introduction

The human immune system can, theoretically, be divided into two highly interactive arms (Roitt 1991). One arm is concerned with adaptive responses to antigen, whilst the other is responsible for non-specific innate immunity, with antigen being defined as molecules which can be classified as either 'self' or 'non-self'. The responses classed as adaptive encompass those in which either T cells (cellular immunity) or B cells (humoral immunity) develop antigen specificity which can increase with time after the initial exposure to antigen. Subsequent lack of exposure to antigen usually leads to a decline in both B and T cell responses unless re-exposure occurs, in which case mechanisms of memory can be evoked. Recognition of non-self is mediated through the major histocompatibility complex genes or MHC. Innate responses, are non-specific but usually have a rapid onset. The initial display of innate immunity often occurs as an antigen penetrates the physical barriers of the body. If foreign material passes beyond the initial barriers or persists at mucosal surfaces, a repertoire of non-specific cells and macromolecules are released to defend the body. Cells such as macrophages, monocytes, neutrophils, killer and natural killer cells recognise general determinants, such as carbohydrate on the invading molecules. The subsequent attachment of the immune cells to the foreign entity via these determinants can lead to its clearance by phagocytosis or lysis. Phagocytosis is enhanced by the deposition of opsonins such as complement components on the surface of the invading organism. IFNs act by mediating the inhibition of growth and replication.

The two different arms of the immune system can interact together to confer specificity to the non-specific mechanisms (Medzhitov et al 1997). The use of antibody is a very good method for achieving this interaction. The presence of a specific antigen on the foreign

cell or organism's surface can lead to antibody attachment via the antigenic recognition or Fab domain. Phagocytic cells such as macrophages and monocytes, as well as cytotoxic cells including natural killer cells, express some or all of the members of the IgG Fc receptor (Fc R) family on their surface (Hogg 1988, Metzger 1991, van de Winkel et al 1993). The non-specific protective cells react with the exposed Fc portion of the antibody, via Fc receptors, and become activated. The result is the removal of the antigen bearing organism though phagocytosis or lysis.

Although the therapeutic use of antibody has been pursued for many years the approach was hampered either by the lack of sufficient antibody or the inability to prevent an anti-globulin response by the recipient against a foreign protein. The use of recombinant non-human monoclonal antibodies can overcome the problem of supply, but the complication of immune cross-reactivity persists. In an attempt to reduce the inherent immunogenicity of non-human antibodies, the cDNA of antibodies such as Campath have been engineered to substitute all possible animal sequences with human equivalents. The resulting humanised cDNA has been used in heterologous expression systems for large scale manufacture. Antibodies produced in this way were initially hoped to pave the way for startling new therapies but unexpected consequences of manufacture soon became apparent.

The first antibody to be humanised and manufactured for clinical trial was Campath-1H. Results from several clinical trials in which the efficacy of Campath-1H was assessed have now been published (Brett et al 1996a and b, Isaacs et al 1996a and b, Dyer et al 1997, Osterborg et al 1996 and 1997, Pawson et al 1997, Bowen et al 1997, Moreau et al 1996). The early clinical trial data reveal that Campath is very efficient at depleting lymphocytes which are CDw52 positive and in the majority of patients the depleted cells return to their original levels within several months of treatment. However, in a few cases, as the lymphocytes are replenished with time, CDw52 negative cells are detected (Brett et al 1996a, Hertenstein et al 1995). On further analysis, the expression defect was shown to involve the mechanism by which the GPI-anchor is attached to CDw52 antigen (Hertenstein et al 1995, Taylor et al 1997) and in these patients all lymphocyte GPI-anchored molecules were affected. A naturally occurring defect of GPI-anchors, paroxysmal nocturnal haemoglobinuria (PNH) is already known and arises as a mutation in an X-linked autosomal gene (Bessler et al 1994), symptoms exhibited include anaemia caused by nocturnal lysis of red cells.

This thesis attempts to address the problems associated with the expression of Campath antibody for therapeutic purposes. It is now acknowledged that engineered antibodies are

influenced by the expression system from which they are derived (Patel et al 1992). Both the host cell line and the method of culture have the ability to create minor changes in post-translational modifications added to the protein backbone. The host cell line in which Campath-1H was expressed at Wellcome Research Laboratories is the Chinese Hamster Ovary (CHO) cell line and for production purposes the cells are cultured in eight thousand litre fermentor vessels in protein-free medium. In Chapter three, I define the mechanisms of action of selected in vitro assays which may constitute the mechanisms by which CHO Campath-1H acts in vivo. Preliminary experimentation with Campath-1H in one of the selected assays, the antibody-dependent cellular cytotoxicity assay or ADCC, had indicated that antibodies with identical antigen specificity exhibited assay variation when expressed and isolated from different animal cells. To explore this further, the humanised cDNA for Campath-1H antibody heavy and light chains were re-isolated and cloned into the alternative Glutamine Synthetase expression system (Bebbington et al 1992). The first part of Chapter four outlines the cloning and expression of Campath-1H in this alternative system and includes both the outline of media development and the comparisons of antibody isolated at various stages in that development. The latter part of Chapter four describes the comparisons of three Campath-1H antibodies derived from different mammalian host cells. In Chapter five, I describe experiments defining CDw52. The mature antigen CDw52 is composed of twelve amino acids which are attached to the cell membrane via a glycosyl-phosphatidyliniositol (GPI)-anchor. The sequence of CDw52 had been published prior to the start of the thesis and subsequent isolation of the antigen cDNA revealed two versions of the sequence (Xia et al 1991, Hale et al 1996). Both isolates contained identical CDw52 sequences in the mature protein, but differences were apparent in the region which controlled the site of GPI-anchor attachment. The differences were limited, at the genetic level, to two base pairs but the positioning of these mutations led to changes in two adjacent amino acids. Both the authentic and alternative isolates of antigen were cloned and expressed in cells in order to have a readily available stock of positive cells for comparison of properties as well as assay development. The expression of the two forms and their comparisons are discussed.

1.3 Antibodies

1.3.1 General antibody structure

A single antibody molecule is composed of four polypeptides, two short light (L) chains and two longer heavy (H) chains (Roitt 1991). The four chains are held together by a series of di-sulphide bridges to form a Y shaped molecule. Within the antibody molecule are two distinct functional areas influencing both specificity and effector functions. The specificity determining area or Fab region contains the amino (NH_2)-termini of one heavy

and one light chain that fold together to form one of two antigen binding sites. Within the Fab portions, the two H and two L chains are both composed of outer variable (V and therefore, VH1 and VL1) and inner constant (C and therefore CH1 and CL1) sequence domains. It is the amino acid sequence within the VH1 and VL1 variable domains which determines the precise antigen specificity. Each variable domain is composed of three short regions of hypervariable sequence called the complementarity determining regions (CDR), interspersed between the more conserved sequences of the variable region. The conserved sequences in both the variable and constant domains contribute to the backbone or framework of the Fab which provides the conformational structure of the antigen binding site.

The portion of the antibody governing the effector function is the Fc region and this is composed of the carboxy (COOH)-termini of both H chains, which are of matched α, γ, δ, ε or μ isotype. Each heavy chain consists of two or three constant domains (CH2, CH3 and CH4), depending on the isotype. IgG is composed of the two γ heavy chains and has two constant domains, CH2 and CH3 (Deisenhofer 1981). The two heavy chains are held together by one or more di-sulphide bridges near to the join or hinge with the Fab region. The length of the hinge determines the flexibility of the two Fab arms and the Fc region in relation to each other and varies with isotype and subclass.

On protein analysis, all normal mature IgG antibody molecules have been demonstrated to contain a carbohydrate component, which is attached to the asparagine residue at position 297 in the CH2 domains of the Fc fragment (Kabat et al 1987). The carbohydrate is added as a whole moiety during a co-translational event (see later text). It is the carbohydrate which provides the antibody molecule with its ultimate conformation and which influences the regions of the hinge and constant region domains that are exposed for interactions with effector cells and macromolecules. Loss or structural change of the attached carbohydrate can alter the function and stability of an antibody molecule and such alterations are observed in diseases such as rheumatoid arthritis (RA) (Parekh et al 1988b).

Structural analysis of IgG antibody molecules by proteolytic digestion with papain releases two Fab fragments and one Fc fragment per molecule. This is a consequence of the cleavage of the antibody, by papain, at a position above the di-sulphide bridges in the hinge region. In contrast pepsin cleavage of IgG antibody gives rise to one $F(ab')_2$, that is two Fab units joined by a disulphide bridge, plus a shorter Fc, or pFc', region. The nomenclature mentioned here will be referred to in the later text when describing genetically modified antibody fragments.

1.3.2 The structural differences between human antibody isotypes

Although the sequence variation between various isotypes of human antibody can be small, the differences seen both between and within isotypes can lead to considerable functional variations (Wawrzynczak 1995). The antibody isotypes IgM and IgE, although not containing a hinge region, do possess an additional CH4 domain (Roitt 1991). Also, several antibody isotypes are capable of forming polymeric structures via the use of an additional tail region at the COOH-terminal of the Fc portion. Isotypes which have this property are IgM, IgA and IgD (Roitt 1991). Polymeric antibodies tend to have high avidity for their epitopes, even when the affinity is low, as a consequence of the increased number of antigen binding sites.

1.3.3 The relationship between human antibody isotype, subclass and complement fixation

Each antibody isotype has distinct functional properties. Some isotypes react with certain cell types through Fc R on the cell surface whilst, once the antibody has bound antigen, others interact with serum components such as complement. IgA and IgM have the ability to activate complement as do some subclasses of IgG. Within normal human serum, the ratios of the various subclasses are 70% IgG1, 20% IgG2, 6% IgG3 and 4% IgG4 (Furakawa and Kobata 1991). IgG myelomas encompass the subclasses IgG1, IgG2, IgG3 and IgG4 which all exhibit large regions of structural homology and so maintain reactivity with anti-IgG antibodies. Minor differences between the subclasses reside within the heavy chains γ 1, 2, 3 and 4 and influence their ability to activate complement. The IgG subclasses IgG1 and IgG3 are the most effective at activation, IgG2 of intermediate activity and IgG4 ineffective (Roitt 1991). The activation of the first complement component, C1q by IgG, occurs by the interaction of the C1q with a characteristic amino acid motif within the CH2 domain of the antibody (Greenwood et al 1993, Brekke et al 1995). The binding and activation of C1q are dependant on the presence of carbohydrate on the asparagine residue in this domain (Leatherbarrow et al 1985, Zhang et al 1994, Wright and Morrison 1994). Also, antibody complexed with antigen has an influence over the C1q binding ability of that antibody in that conformational restraints are imposed on the antibody by the bound antigen. An additional effect of antigen density and distribution is also imposed on the antibodies ability to activate complement due to the likelihood of the complement binding multiple sites of adjacent Fc regions.

1.3.4 Rat/Mouse antibody subclasses in relation to human subclasses

Mouse IgG subclasses IgG1, IgG2a, IgG2b and IgG3 are classified in a similar way to the human IgG subclasses IgG1, IgG2, IgG3 and IgG4. The rat subclasses are IgG1, IgG2a,

IgG2b and IgG2c. All antibody subclasses have different inter- and intra-species amino acid sequences, distribution of di-sulphide bonds and physiological properties, but comparisons can be made on a functional basis (Wawrzynczak 1995). Mouse IgG2a, 2b and IgG3 and rat IgG1, IgG2a and 2b can be equated with human IgG1, 2 and 3 in that they activate complement. Also both mouse IgG2a and rat IgG2b antibodies are able to interact with human IgG Fc R and thus can trigger mechanisms such as phagocytosis via human effector cells (Hale et al 1985).

1.3.5 Human IgG subclass structure and Fc receptor binding

The structural differences between IgG subclasses include both different amino acids and interchain disulphide bridging which contribute to alterations in biological activity (Roitt 1991, Brekke et al 1995). IgG1 is able to bind Fc R adequately and is distinct in its form of di-sulphide bridging between the H and L chain. Most subclasses exhibit linkage between a cysteine residue in the COOH-terminus of the L chain with a cysteine residue sited between the VH and CH1 domain. The site of linkage in IgG1 is via a cysteine in the hinge region located between the CH1 and CH2 domains. IgG3 contains an extended hinge region which seems to be a quadruplication of the IgG1 hinge and, whilst it binds Fc R as well as IgG1, it is the only subclass to demonstrate self aggregation. The extended hinge is not required for Fc R mediated activity but the di-sulphide bridging is essential (Aase et al 1993). IgG4, like IgG1, contains two inter-chain di-sulphide bridges in the hinge region and is not thought to bind to Fc R on monocytes in vitro. However, this is disputed for in vivo use (Isaacs et al 1996c). IgG2, contains four disulphide bridges in the hinge region and binds to Fc R but not with high affinity.

1.3.6 IgG antibody glycosylation

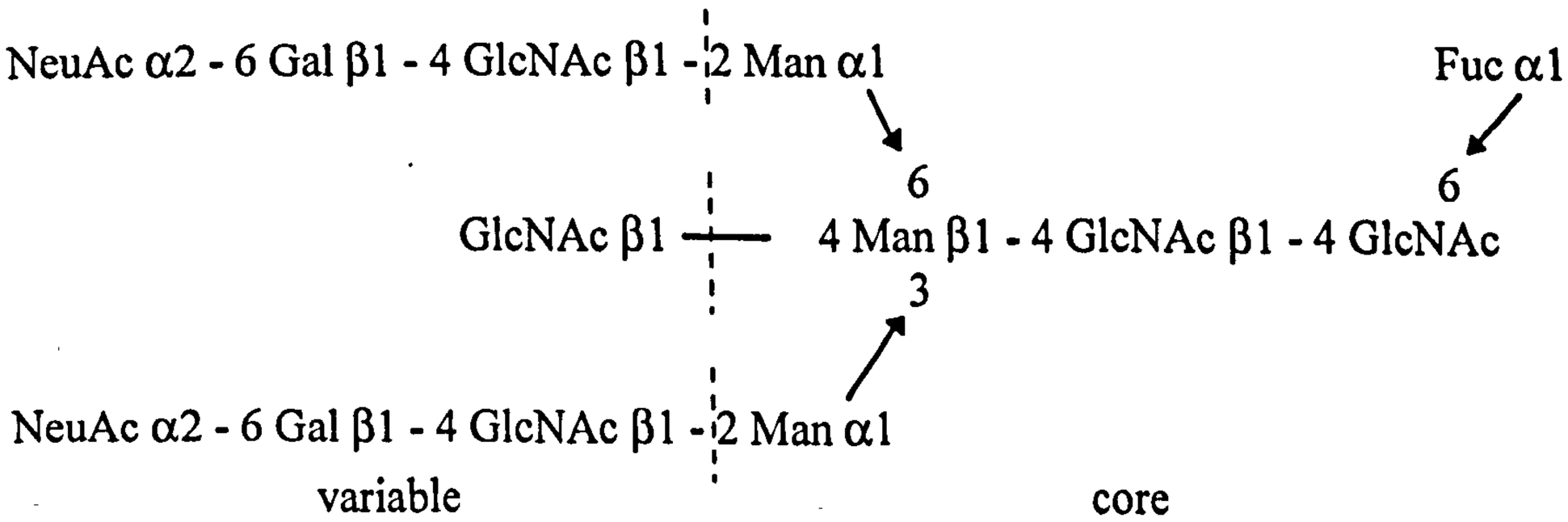
Mutational analysis of the protein backbone of IgG antibody indicates that the residue for carbohydrate attachment in the Fc region is the asparagine residue at position 297 (Kabat et al 1987). This residue is within CH2, which is limited conformationally by the hinge di-sulphide bonds at the NH_2-terminus and by the form of linkage to the CH3 domain at the COOH-terminus (Rudd et al 1991). The carbohydrate moiety attached to the asparagine residue can be composed of a variety of structures or isoforms, which may vary according to the species from which the antibody is derived (Parekh et al 1989, Rademacher et al 1988), the disease state of the host species (Parekh et al 1988b) and, if the antibody is recombinant, the source and culture conditions of the host cell line in which it is expressed (Paulson 1989, Patel et al 1992, Goochee et al 1990). The CH2 domains are unlike the other domains in that they lack extensive protein-protein interactions and rely on the carbohydrate residues on each heavy chain to maintain conformation. Loss of the carbohydrate also influences biological activity (Nose and

Wigzell 1983, Leatherbarrow and Dwek 1983, Leatherbarrow et al 1985, Elbien 1991, Furukawa and Kobata 1991, Boyd et al 1995, Lifely et al 1995). Glycosylation sites can also be present in the variable regions of antibodies if in the correct context of asparagine-X-serine/threonine.

1.3.6.1 Carbohydrate structure

The carbohydrate isolated from IgG Fc has been demonstrated to be of bi-antennary complex-type structure (Parekh et al 1989, Rademacher et al 1983), which is one of three possible classes of N-linked oligosaccharide. The oligosaccharides, when isolated, are rarely the same on both heavy chains and the monosaccharide combinations feasible within the oligosaccharides are numerous. At least thirty different oligosaccharide structures are possible on each site (Parekh et al 1985, Rudd et al 1991). Each oligosaccharide is manufactured prior to addition and is transferred, co-translationally, to the site of attachment onto the nascent polypeptide (Parekh et al 1989, Goochee et al 1990). Oligosaccharide moieties are composed of a core structure of two N-acetyl glucosamine (GlcNAc) residues often with one fucose residue on the inner GlcNAc (86% of normal serum) and three mannose residues (Rademacher et al 1983, Rudd et al 1991). Additional outer arm residues of either none, one or two GlcNAc, none, one or two galactose residues, and possibly none, one or two sialic acid residues can also be present. Within normal human serum the ratios of none, one and two sialylated oligosaccharides tends to remain constant at 76:18:6 (Furakawa and Kobata 1991). Extra GlcNAc residues can also bisect the two armed core and lead to further complexity of the structure (see Fig.1.1). These are seen in 18% of normal human serum but not in mouse sera (Furakawa and Kobata 1991).

Figure 1.1 A diagrammatic representation of the possible carbohydrate structure associated with IgG Fc. NeuAc represents sialic acid, Gal represents galactose, GlcNAc represents N-acetyl glucosamine, Man represents mannose and Fuc represents fucose. α and β refer to the linkage types.



Within each oligosaccharide entity it is possible to identify defined secondary structures and the bi-antennary conformation arises from the two glycosidic linkages between the three mannose residues (Rudd et al 1991). The α(1-6) linkage, which forms one arm of the bi-antennary structure, can assume one of two conformations which allows a certain flexibility. It is this arm, on each of the two oligosaccharides, which interacts with the antibody backbone on the opposite heavy chain. Once the interaction has occurred the flexibility of the sugars is then constrained. The α(1-3) linkage is more rigid and it is this arm, on one of the sugars, that interacts with the core of the opposing oligosaccharide. To enable this to occur, the terminal sialic acid and galactose residues of this arm are absent, exposing the GlcNAc for interactions. The remaining free α(1-3) arm of the second oligosaccharide extends outwards between the antibody domains with no apparent steric restraints on its length. To enable comparisons between oligosaccharide structures to be made, a universal numbering system for the residues has been devised (described in Lifely et al 1995). Each residue, from the outermost inwards, has a number referring to the number of those residues, such that 004301 represents no terminal sialic residues, no galactose residues, four GlcNAc, three mannose, no bisecting GlcNAc and one core fucose. The example in figure 1.1 would be represented by 224311.

1.3.6.2 The biosynthesis and addition of N-linked carbohydrate

A precursor oligosaccharide is synthesised by the sequential addition of the sugars GlcNAc, mannose and glucose, from their activated glycosyl donors, to the lipid carrier dolichol-P that results in a Glc3Man9GlcNAc2-pyrophosphoryl-dolichol lipid-linked oligosaccharide moiety (Elbein 1991). The reactions are all catalysed by membrane-bound glycosyl-transferases and occur in the endoplasmic reticulum (ER) prior to processing (Goochee et al 1990). The antibiotic tunicamycin blocks the action of GlcNAc transferase, the enzyme responsible for the transfer of GlcNAc-1-phosphate (P) from uridine di-phosphate (UDP)-GlcNAc to dolichol-P and thus prevents the formation of the precursor (Goochee et al 1990). The precursor structure is a common intermediate of all three classes of N-linked oligosaccharides and the final oligosaccharides are synthesised from the intermediate by exoglycosidase trimming of the oligosaccharides whilst still in the ER. The immature N-linked oligosaccharides are attached covalently, via a N-glycosidic bond, to the NH_2 group of asparagine when displayed in the context of asparagine-X-serine/threonine. Not all asparagine residues found in the correct context are glycosylated and it would seem necessary for the residue to be in an exposed region such as a β-turn (Elbein 1991). Once attached and whilst the polypeptide continues to be synthesised, the oligosaccharide is processed further by glucosidases 1 and 2 to remove the three glucose residues. Mannosidases (alpha- and endo-) further delete either one or

two of the mannose residues before the protein is transferred to the Golgi apparatus. On transfer, the enzyme mannosidase 1 removes between one and four mannose residues to give Man5GlcNAc2-protein prior to the addition of a GlcNAc residue from its UDP-GlcNAc donor. The excess mannose residues are now removed by the action of mannosidase 2 and the oligosaccharide elongated to its final form. This is achieved by the addition of various sugars utilising specific glycosyl transferases located in the trans-Golgi.

1.3.6.3 Effects of glycosylation on polypeptides

Oligosaccharide additions to polypeptides such as antibodies are acknowledged to have profound effects in that they can alter their biological properties (Leatherbarrow et al 1985, Nose and Wigzell 1983) such as Fc binding (Jefferis et al 1995) and complement activation (Zhang and Lachmann 1994). Evidence (Elbein 1991) from the use of either site directed mutagenesis, enzymatic removal or glycosidase inhibitors, has also shown that the presence or absence of carbohydrate can alter protein conformation, thermal stability, charge and solubility as well as location and distribution. In vivo studies on the immunogenicity of glycoproteins (Schauer 1988) have demonstrated that terminal sialylation of proteins reduces their immunogenicity and it is interesting to note that during pregnancy, the amount of sialic acid and galactose residues found on antibody increases (Rook et al 1991). In the absence of terminal sialic acid, proteins are rapidly cleared from circulation by hepatocytes and macrophages via carbohydrate specific receptors (Ashwell and Harford 1982). In rheumatoid arthritis (RA) (Parekh et al 1985), the degree of terminal sialylation and galactosylation of synovial fluid IgG decreases and the extent of reduction can be shown to correlate with severity of disease. Pregnancy can reverse the outcome of this reduction and give temporary relief from symptoms (Rudd et al 1991). When referring specifically to the administration of antibodies for therapy, the types of oligosaccharide present are also critical. Within human serum there is approximately 1% of an antibody which is specific to Gal(α1-3)Gal residues, outer arm oligosaccharides found on antibodies from New World monkeys and non-primate mammals such as mice (Galili et al 1988, Thall and Galili 1990, Galili 1993). The presence of this antibody enhances the clearance of mouse antibodies administered systemically and drastically limits their use (Borrebaeck et al 1993). Glycoproteins expressed in yeast or insect cells may also be rapidly cleared because both of these systems produce carbohydrates with terminal mannose residues (Paulson 1989). When placed into either complement lysis or ADCC assays, antibodies expressed in yeast do not activate complement but still exhibit ADCC activity (Horwitz et al 1988).

The cells in which polypeptides are expressed can also influence the glycosylation and this has been demonstrated for many proteins. A well documented example is the Thy-1 molecule in the rat which, whilst maintaining its protein backbone, is found to display very different glycosylation patterns in the forms isolated from either the brain or thymus (Parekh et al 1987).

1.3.6.4 In vitro factors affecting carbohydrate composition

Pregnancy, RA and other physical conditions are well documented in terms of their association with changes in the glycosylation of cellular proteins (Muramatsu 1993) and antibody Fc domains (Rudd et al 1991, Parekh et al 1985, Knight 1989, Rook et al 1994). In vitro conditions are also able to influence carbohydrate addition. Lund and colleagues (1993) provided evidence that the protein backbone of antibody produced in vitro imposed some restraint on the oligosaccharide content in that, under controlled conditions, the preference for sites of galactosylation correlated with isotype. Glucose starvation during the culture of cells such as CHO or mouse 3T3 cells can lead to either aberrant glycosylation (Gershman and Robbins 1981) or more dramatically, total lack of glycosylation i.e. in monoclonal antibody light chain synthesis from MOPC-46 cells (Stark and Heath 1979). Hormones such as retinoic acid have been demonstrated to play an important role on the differentiation state of epithelial cells in vivo (Wolf 1984), whilst the hormone can exhibit an influence on glycoprotein oligosaccharide processing in vitro (Chan and Wolf 1987). Another known cause of altered antibody glycosylation is the method of protein production (Anderson et al 1985) and it was noted that IgM produced from different harvests of mouse ascites fluid differed in sialic acid content whilst antibody produced in cell culture exhibited incomplete oligosaccharide processing. This observation has been confirmed by Lund et al (1993) who clearly demonstrated that culture conditions influenced the extent of galactosylation, with the highest levels monitored in cells cultured as monolayers. They also showed that the cell type in which the antibody was produced was critical. In these experiments the carbohydrate profiles of human/mouse and mouse immunoglobulins expressed in mouse J558L cells were compared with similar immunoglobulins from CHO cells. Only the antibody from the mouse J558L cells was found to contain Gal(α1-3)Gal linkages. Host cell lines themselves possess different arrays of glycosyl-transferases as found in CHO (Hokke et al 1990) and HeLa cells (Nilsson et al 1993). Evidence for the influence of tissue culture method was supplied by Patel (et al 1992), whose data inferred that not only culture method but also media could alter the oligosaccharide profile of a protein. They compared IgG1 monoclonal antibody produced from cells grown in serum-free or serum containing medium with those isolated from ascites. The differences were that terminal sialylation and galactosylation varied. The antibody from ascites contained 98%

asialylated oligosaccharides whilst cells cultured in medium produced antibody with 81% asialo, 15% mono- and 4% di-sialylated molecules in the presence of serum and 71% asialo, 18% mono- and 11% di-sialylated oligosaccharides when cultured in serum-free medium. Both of the cell culture methods produce antibody with similar ratios to human IgG sialylation (76:18:6, Furakawa and Kobata 1991). When the galactose content was compared, antibody from cells cultured in serum was found to be below that of the other two forms of antibody. These findings correlate with the data produced by Lifely (Lifely et al 1995) and those described in Chapter five, which indicate that the method of culture, species of cell line and composition of medium does indeed influence antibody glycosylation patterns.

1.3.6.5 The consequences of the removal of carbohydrate from IgG

Rademacher (et al 1988) observed that when placed into in vitro assays in comparison with intact antibodies, a- or de-glycosylated antibodies demonstrated a divergence in efficiency. In the ADCC experiments, deglycosylated antibodies failed to activate effector mechanisms whilst complement activation and C1q, protein A and antigen binding were not altered. As Rademacher pointed out, this reflected an effect on "all interactions with cellular bound receptors, whereas fluid-phase reactions are unaffected". Deglycosylation of mouse monoclonal antibodies by tunicamycin treatment of hybridoma producing cell lines, revealed no loss of staphylcoccal protein A binding (Leatherbarrow and Dwek 1983) whilst a reduction in their specific activity in terms of Fc R recognition and complement activation was observed (Leatherbarrow et al 1985). The latter were found to activate ADCC less efficiently. These results are entirely in line with more recent data (Boyd et al 1995) that showed that deglycosylation of Campath-1H led to a loss of both ADCC and complement lysis whilst the antibody retained antigen and protein A binding activity. The same group illustrated that the removal of the terminal sialic acids was ineffectual, whilst the subsequent removal of the Campath galactose residues reduced activity in complement lysis assays but not ADCC. Pound and his colleagues (1993a) have demonstrated the ability of aglycosylated IgG3 to trigger the human phagocyte respiratory burst.

The removal of carbohydrate is also thought to affect the catabolism of the protein and thus clearance rate (Wawrzynczak et al 1992). When intact or aglycosylated IgG2b mouse antibody was administered to rats, and measured by both enzyme-linked immunosorbent assay (ELISA) and radiolabel tagging, the antibody half-life of the latter antibody was reduced from 7.4 days to 4.8 days.

1.4 The use of antibodies in therapy

1.4.1 The early history

The idea of antibodies being used therapeutically (review by Riethmuller et al 1992) was first postulated in the late 1800s by Metchnikoff (Metchnikoff 1899) who described the possible use of antibodies in vivo. This concept was fostered by Paul Ehrlich who envisaged the antibody molecules as 'magic bullets or immunobodies'. He recognised that they were capable of both antigen recognition and complement activation and thus of potential use as targeting and blocking agents. In suggesting this approach, Ehrlich also anticipated the anti-idiotype network. Perlmann in 1959 (Perlmann 1959), took antibody therapy one step further by demonstrating that not only could antibodies block, but they could also activate as indicated by their ability to stimulate sea urchin eggs to divide. His data led to the early use of antibodies as stimulants of lymphocytes by the application of anti-lymphocyte globulin (ALG). A major step forward in the development of antibody technology was taken by Waksman and colleagues in the 1960s (Waksman et al 1961), who utilised the property of antibody binding to specific determinants on cells to block immune reactions in vivo and soon manufacturers were producing ALG to be used as an immunosuppressive reagent for organ transplantation. In terms of mechanism of action it was not clear at that time whether it was necessary to remove lymphocytes or merely suppress their action. It was difficult to prove that depletion through complement-mediated or Fc-mediated lysis could be separated from suppression. Riethmuller (Riethmuller et al 1968) clarified the issue by demonstrating that both F(ab') and $F(ab')_2$ fragments of antibody failed to induce immunosuppression but the requirement for lysis remained not proven. It was only when in vivo tests on monkeys became routine, that the involvement of the Fc portion in effector activation was clarified.

1.4.2 Monoclonal antibody technology

It was not until the novel hybridoma technology of Kohler and Milstein (Kohler and Milstein 1975) was established in 1975, that the large scale manufacture of antibodies for clinical use became possible. Up until this major leap forward in methodology the use of antibodies in therapy had been severely limited to treatment of patients with polyclonal serums raised in animals as demonstrated by the early work of von Behring and Kitasato (1890). By the use of hybridoma techniques, it was possible to consider the generation of specific monoclonal antibodies of rat, mouse and even human origin. Some of the first antibodies generated by the new technique were anti-lymphocytic, i.e. OKT 3, which is a mouse anti-human CD3 monoclonal antibody (Ortho 1985). Campath-1M (Hale et al 1983) was another monoclonal antibody, derived from rats immunised with human T

cells, which was utilised for the in vitro depletion of lymphocytes from graft bone marrow.

The mouse monoclonal OKT3 was first placed into trial and reported in 1985 by the Ortho multicentre study group (Ortho 1985) whilst Campath-1M was initially utilised in vitro and reported in 1983 (Hale et al 1983) and subsequently administered in vivo in 1989 (Dyer et al 1989). Since that time however, several problems associated with the utilisation of animal cell derived antibodies have been encountered .

1.4.3 The problems encountered with the use of animal cell derived antibodies

1.4.3.1 Monoclonal isotypes

The process of derivation of a monoclonal antibody usually involves the fusion of an immunised rat or mouse spleen cell with cells from a rodent B cell tumour line (Kohler and Milstein 1975). The resulting hybridomas are able to secrete antibody in high quantities and by selection in defined media, unfused tumour cells are killed and unconjugated spleen cells die. On dilution cloning and characterisation, antibody producing clones of distinct specificity can be obtained. The initial immunisation schedule of the animals governs the final isotype and specificity of the antibody. In the early stages, the majority of monoclonal clones were of the IgM isotype. This outcome was due to the lack of T cell recognition sites on many of the immunogens utilised i.e. peptides and resulted in the lack of co-operation between T and B cells when immunisation occurred. Unfortunately, only the mouse IgG2a and the rat IgG2b isotypes (Hale et al 1985) are able to engage human effector cell mechanisms, whilst both IgM and IgGs activate complement. This problem has now been overcome by the selective and rational choice of immunogen.

1.4.3.2 Human monoclonal production

Attempts to produce human monoclonals have been fraught with difficulties. The only reliable source of immunised cells are those from the peripheral blood and the route of immunisation is generally through natural infection, cancer or autoimmunity. In vivo immunisation specifically for the purpose of raising monoclonals is thought of as unethical whilst in vitro immunisation of human cells (Borrebaeck 1989) is possible but it generally produces antibody of the IgM isotype which is of low affinity and diversity. To immortalise the immune cells, it has proved difficult to find human tumour cell lines with similar properties to those of the rodent lines. Alternative strategies have involved fusion to either mouse cell myelomas or to human/mouse heteromyelomas (Hancock et al 1988). Transformation of the immune cells with Epstein-Barr virus (EBV) as a means of

immortalisation has also been attempted (Thompson et al 1986, Borrebaeck 1989) but the levels of antibody produced are low and the resulting cell lines unstable.

1.4.3.3 Human Fc receptor polymorphisms

In vitro and in vivo studies utilising mouse and rat derived antibodies to mediate human effector functions indicate that not all rodent isotypes are able to elicit identical immune responses (Hale et al 1987, Dyer et al 1989). This is also true for interaction of human Fc receptors with human antibody. The reason for the variation is IgG Fc R polymorphism. It is known that both human Fc RII and RIII exhibit polymorphic forms which interact with human IgG in different ways. Fc RIIa is found on phagocytes such as monocytes and neutrophils and is the only receptor which can interact with human IgG2 raised in response to bacterial capsular polysaccharides and therefore influences bacterial susceptibility. There are two Fc RIIa alleles, R131 and H131, with the former interacting poorly with complexed human IgG2 whilst the latter H131 binds well (Sanders et al 1995). These qualities are reflected in the ability of cells bearing the receptors to phagocytose human IgG2 opsonised bacteria. In reverse, Fc R11a-H131 donors poorly phagocytose bacteria opsonised with mouse IgG1 whilst the opposite is true for the -R131 form. Mouse IgG2b antibody was found to interact only with Fc R11a-H131 (Haagen et al, 1995). There are similar polymorphisms for IgG binding found with Fc RIIIa, -158V or F, on NK cells (Koene et al 1997) and Fc RIIIb, a GPI-anchored receptor also on neutrophils, with the alleles termed Fc RIIIb-NA1 and NA2 (Salmon et al 1992) These receptor polymorphisms all strongly influence individuals responses to bacterial infection as well as to immunotherapy.

1.4.3.4 The half-life of animal derived antibodies

Studies on the therapeutic use of antibodies derived from sources other than man have demonstrated that clearance rate from the circulation is faster than that of normal human immunoglobulin. Mouse IgG administered to humans exhibits a half-life of 7-8 days (Wawrzynczak et al 1992) whilst human IgG subclasses 1, 2 and 4 have a half-life of approximately three weeks. Human IgG3 has a shorter half-life of one week as a consequence of its extended hinge region which leaves it prone to proteolytic degradation. The half-life of human IgM is also less than the majority of IgG subclasses because its large size limits its ability to cross out of the vascular system. Attempts to define the catabolic site on IgG have identified residues within the CH2-CH3 junction (Kim et al 1994). The carbohydrate additions on the human IgG CH2 domain protect the antibody from catabolism and the loss of these enhance its degradation leading to a reduction in half-life to 4.8 days (Wawrzynczak et al 1992). Antibodies derived from animals, when injected into humans, can induce immune recognition which leads to the

formation of antibody/antigen complexes. As stated previously, 1% of human IgG is directed against the Gal(α1-3)Gal linkage present in mouse derived antibodies (Galili et al 1988, Thall and Galili 1990, Galili 1993). If not cleared by the reticulo-endothelial system (RES) quickly, the complexes can become deposited on basement membranes and elicit inflammation via the activation of complement.

1.4.3.5 Human anti-species response

When monoclonal antibodies were originally considered as therapeutic agents the potential problems of repeated use in vivo were foreseen from experience with polyclonal antibodies. The first experiments documented with monoclonals such as Campath-1M were constrained to in vitro studies in which the antibody was utilised in conjunction with complement to deplete cell populations of specific antigen bearing subsets (Hale et al 1983). A year later in 1984, Cobbold administered the monoclonal anti-mouse equivalent of the human Leu3/T4 marker in vivo and achieved good depletion (Cobbold et al 1984). However after several trial situations in which the earlier in vitro experiments were followed by multiple in vivo treatments, it became evident that patients developed immune responses to the foreign material. In clinical cases where monoclonal antibody was only administered once, this was not considered to be a problem. But, when the possibility of multiple treatments became a reality, this was a potential hazard. Experimentally, mice were found to respond to multiple administration of antibody by producing either anti-allotypic or anti-idiotypic antibodies of their own (Benjamin et al 1986a and b, with anti-Leu3/T4 antibody) which eventually lead to inactivation of the administered antibody. Examples of these responses in the early Campath-1G trials were found in two of the twenty-nine patients (Dyer et al 1990b). Methods to overcome the problem were actively sought and several techniques evolved (Mountain and Adair 1992). Probably the most significant solution arose from the studies of Winter (1989) who devised the strategy of 'Humanisation.'

1.4.3.6 Bio-availability of target

When investigating the utilisation of antibody immunotherapy several factors should be considered prior to treatment (Chester and Hawkins 1995). For the majority of cases, it is essential that the antibody to be utilised must demonstrate non-cross reactivity with normal tissue, whilst the antigen or epitope to which it is raised must be expressed to a sufficiently high level on the abnormal cell to provide a good target. The choice of antibody isotype must also reflect the ultimate use of the reagent, in that if activation of effector cells is required the IgG1 or IgG3 isotypes are more suitable than IgG4 which does not bind Fc receptors. However, if the therapy involves the blocking of antigens or capture of cytokines, Fab fragments, IgG4 (Hutchins et al 1995) or Fc-mutated antibodies

would be more advantageous. When treating solid tumours, rather than blood borne disease, penetration of the tissue must be optimised and is best achieved by the use of small fragments of antibody. These, in addition, can be coupled to toxic (Mansfield et al 1997a and b) or radioactive compounds to ensure maximum by-stander killing.

1.5 Mechanisms utilised to alleviate responses to animal derived antibody products.

1.5.1 Chimeric antibodies

Using molecular biological techniques, the rodent antibody variable heavy and light chain domains are isolated and recloned into vectors containing the coding sequences for the constant domains of the human Ig of the isotype required. This process retains the antigen specificity characteristics of the mouse antibody whilst incorporating the human effector functions. Horwitz and colleagues (1988) utilised yeast cells to express products of this type and were able to demonstrate target cancer cell binding and ADCC activity. Sometimes however, the human Fc sequences can decrease the affinity of the Fab region for the antigen, because of structural restraints. If non-human primate antibodies are chimerised in this manner the term used to describe the product is primatised (Newman et al 1992). For both techniques, the foreign amino acid sequences can still give rise to both anti-idiotypic and anti-allotypic responses as they constitute about 30% of the total Ig structure.

1.5.2 Humanised antibodies

The CDR regions of the rodent antibody variable domains are rescued by polymerase chain reaction (PCR) and replaced into the nearest match of human framework possible (Winter and Milstein 1991, Winter and Harris 1993). Extensive remodelling of the variable regions may be required, to ensure the correct conformation of the CDR, as the removal of mouse framework sequences can alter antigen binding properties. The substitution of framework sequences with mouse sequence to reshape the Fab region may be required to retain or improve the original binding pattern. If human sequences are utilised to reshape the structure it is called resurfacing (Roguska et al 1994). Concurrently, the constant domains of the rodent antibody are also replaced with those of a desired human isotype and subclass to ensure correct effector functions. The technique of humanising antibodies was originally established as a means of bypassing the patient anti-species reaction but anti-idiotypic reactions can still occur as the foreign sequence still constitutes 3% of the Ig structure.

1.5.3 Recombinant antibody fragments

Two of the many problems associated with the engineering of whole rodent antibodies is the limitation imposed by the need to utilise existing antibodies as template and the inability of prokaryotic expression systems to process the antibodies correctly in terms of glycosylation. For antibody fragments composed of Fab or F(ab') components only, the expression host is not so important because generally these regions are not glycosylated.

1.5.3.1 Fab and F(ab') fragments

The expression of the light chain in combination with the VH and CH1 domains is achieved by cloning the rodent antibody sequences into vectors containing either bacterial sequences which target the product to the periplasm of the host *Escherichia coli* or yeast sequences which allow secretion (Horwitz et al 1988). The signal sequences are cleaved naturally and the two chains associate and form both inter- and intra- di-sulphide bonds in the periplasm. $F(ab')_2$ fragments can also be generated in this way if the cloned VH and CH1 region include extra COOH-terminal sequences corresponding to either the hinge region or a sequence which includes one or more cysteine residues. When expressed, the two chains re-associate to form the $F(ab')_2$ structure.

1.5.3.2 Fv fragments

Fragments of antibody smaller than the Fab and Fab' portions still retain antigen specificity and the isolated VH and VL domains can be cloned into bacterial vectors for expression (Glockschuber et al 1990b). It is usual to link the two in some way because they lack the natural sites for di-sulphide bridging and therefore form only weak non-covalent bonds between themselves. The type of links formed can be simple amino acid changes within the actual fragments to create di-sulphide bond sites, or involve the introduction of spacers between the heavy and light chain sequences which allow the two fragments to fold back onto themselves once expressed. The latter results in a structure referred to as a single-chain Fv or scFv (Rumbley et al 1993). The scFv fragments have been demonstrated to be at least ten times more stable than the isolated domains (Glockschuber et al 1990a and 1992).

1.5.3.3 Single domain antibodies

Individual domains of the heavy and light chains can recognise and bind antigen (Davies and Reichmann 1995). It is possible to express either VH or VL domains in bacteria although VH tend to demonstrate higher diversity due to earlier recombination events. As the domains are not protected by the interaction with the corresponding partner domain they demonstrate greater susceptibility to degradation but camelization can improve the stability (Davies and Reichmann 1996).

1.5.3.4 Multiple specificity antibodies

Antigen specificity of an antibody can be diversified by the artificial linking of two or more F(ab') fragments from different antibodies (Holliger et al 1993 and 1996, Malygin et al 1994, van de Winkel et al 1997). The isolated F(ab') fragments are treated chemically in such a way as to form either di-sulphide or thio-ester bonds which link them together. More complex interactions involving more than two fragments are possible but require the use of polyfunctional crosslinkers to allow conjugation. The therapeutic use of these complexes can be extended by the addition of one or more human Fc regions to stimulate effector functions in vivo (Ellis et al 1995) or by genetic modification of Fc terminal structures to enable multiple-domain antibodies to be constructed (Greenwood et al 1994).

By the utilisation of bacterial expression systems it is possible to create recombinant bi-specific Fab fragments. As for the single Fab, the VH and VL and CH1 sequences are cloned but at the COOH-terminus the sequence for one half of a leucine zipper, such as *fos* or *jun*, is added (Roitt 1991). When two Fab regions are co-expressed in bacteria the two halves of the leucine zipper associate and create the Fab dimer. Similar methodology which introduces inherent links between two different scFv allows dimeric scFv to be formed on co-expression in the host. The leucine zipper technique can be applied to scFV but the most common forms of linkage are of the physical type in which long polypeptide linkers join the COOH-terminal of one scFv construct with the NH_2-terminal of the second. An alternative approach is to express all four regions together with peptide links between the VH of one antibody with the VL of the other. When produced, the VH and VL of the authentic scFV re-associate but as a dimer in conjunction with the second scFv. The latter are termed diabodies (Holliger et al 1993 and 1996).

1.5.3.5 Phage display libraries

Experimentation in the 1990s (Hoogenboom et al 1991, Clackson et al 1991, Williams et al 1993, Winter and Winter 1994, Kettleborough et al 1994, Nissim et al 1994) explored the possibility of expressing various antibody domains in bacterial filamentous phage whose products were not only formed but displayed on the phage surface. The technique has the advantage that it anchors the antibody fragments for further screening and amplification procedures. The technique requires the antibody fragments to be cloned as fusions to a phage coat protein, either pVIII or pIII. The former protein is present in the phage in 2000-3000 copies and constitutes the majority of the phage coat, whereas pIII is only present in 3-5 copies and is expressed only on the tip of the phage particles. The process of joining the antibody fragment to the carboxy-terminus of either pVIII or pIII

ablates their infectivity, but this can be overcome by utilising a phagemid partner, which expresses wild type pVIII or pIII, to infect bacteria. The diversity of natural V domains for possible use in this system can be enhanced artificially by the use of not only domains from immunised donors but also those from naive individuals or even synthetic randomised gene sequences (Davies and Reichmann 1995 and 1996).

1.5.3.6 Intracellular antibodies

The recent application of expressing antibody within transfected cells has opened the possibility of direct treatment of patients without incurring the injection of antibody (Richardson and Marasco 1995, Biocca and Cattaneo 1995). The natural NH_2-terminal signal sequence of an antibody directs its transport to the plasma membrane. If this is removed or replaced with a hydrophilic signal sequence, the resulting antibody can be re-directed to the cell cytosol. The addition of alternative signal sequences can further re-direct the antibody to the nucleus or other cellular organelles. The technique at present has only been utilised to express antibody or fragments of antibody within laboratory transfected cells and so the problem of targeting in patients has not been fully explored.

1.6 Derivation of the Campath series of antibodies

1.6.1 The monoclonal antibodies

Campath is the trade name of the Glaxo-Wellcome monoclonal antibody product which recognises the antigen CDw52. When first isolated as monoclonal hydridoma fusions in the early 1980s, the antibodies produced were of either IgM (Campath-1), IgG2a (YTH34.5) or IgG2c (YTH86.1) isotype. As therapeutic agents they were useful in vitro but of limited potential for in vivo utilisation because of their isotypes (Hale et al 1985). The IgM antibody was utilised in several trials in which human bone marrow was depleted of lymphocytes prior to transplantation into immunosuppressed patients (Hale et al 1983, Morgan et al 1986). However, in the former trial the depletion was only transient and some of the patients developed graft versus host disease (GVHD).

1.6.2 The class switch of IgG2a to IgG2b

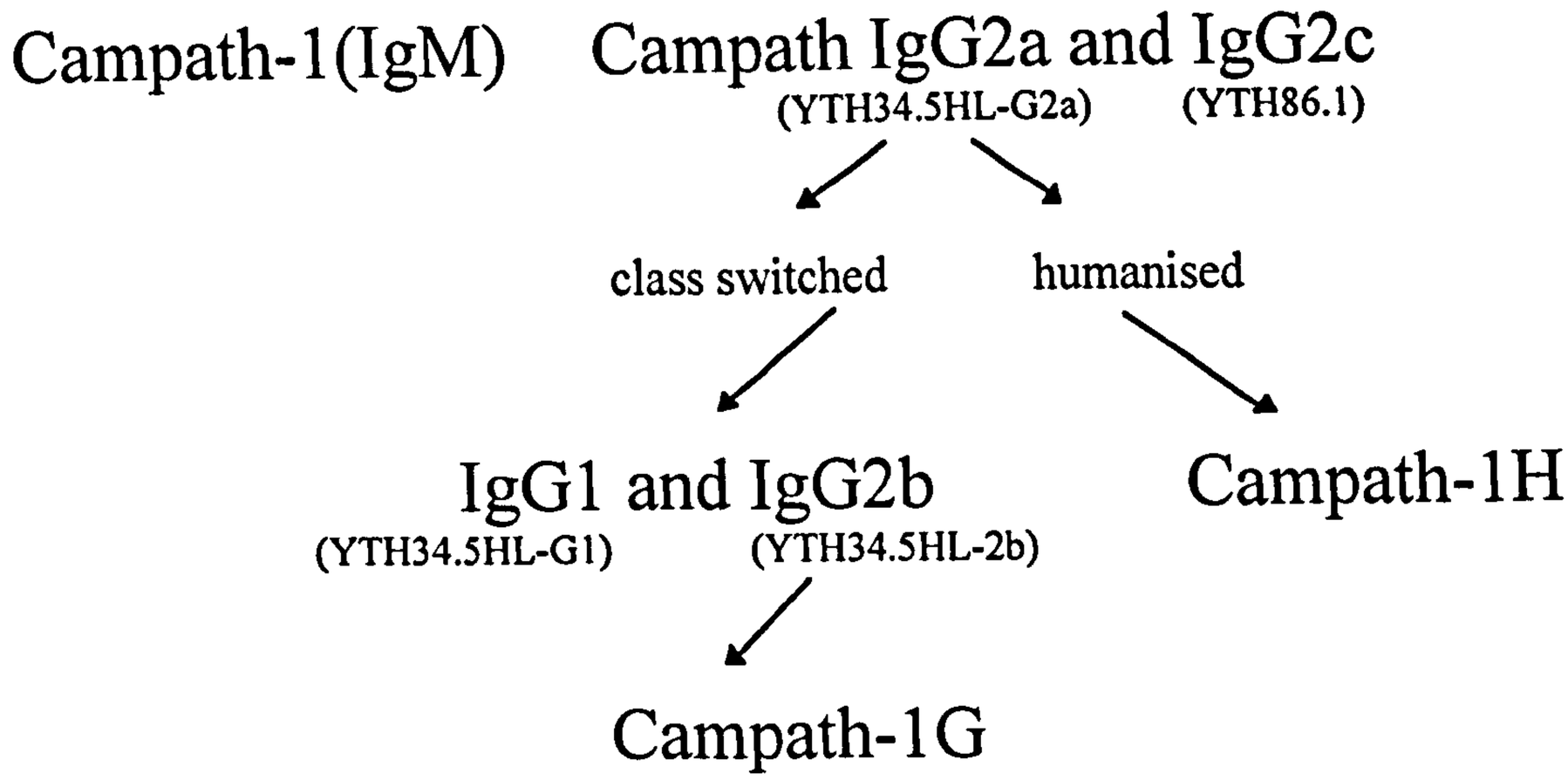
To develop a rat antibody of different isotype which could be utilised in vivo i.e. IgG2b, the IgG2a producing hydridoma line YTH34.5HL-G2a was subjected to long-term culture which favoured class switching (Hale et al 1987). The resultant hydridoma lines raised from the process produced either IgG1 (YTH34.5HL-G1) or IgG2b (YTH34.5HL-

2b) antibody after 8-9 months in culture, but at a very low frequency of 0.8-0.9x10^{-8}/cell/generation. When the resulting antibodies were compared in biological assays, both IgG2a and IgG2b as well as IgG1 were able to lyse radiolabelled human mononuclear cells in the presence of complement (Hale et al 1987). However IgG2a and 2b were of equivalent activity whilst IgG1 was less efficient. Assays demonstrating the binding of C1q by the antibodies indicated that IgG2b was three times more efficient than IgG2a whilst in ADCC assays, IgG2b was the most active. Consequently the IgG2b isotype was selected for continued development as a therapeutic reagent and in an exploratory trial IgM (Campath-1), IgG2a (YTH34.5) and the IgG2b antibody referred to as Campath-1G, was administered therapeutically in vivo (Dyer et al 1989).

1.6.3 Humanisation strategy for Campath-1G

The use of Campath-1G in vivo raised the possibility of anti-rodent responses occurring in patients during repeated infusions of rodent monoclonal antibodies. To avoid these consequences, a strategy of molecular alterations was devised and performed for the rat antibody in three stages (Reichmann et al 1988). The monoclonal hydridoma expressing Campath IgG2a, or YTH 34.5, was utilised as the donor of the variable region CDRs for restructuring of the antibody. The heavy chain CDRs were isolated and placed, as stage one, into the NEW human heavy chain framework after noting the alteration of residues 41 and 45. In most heavy chains these are represented by proline and leucine respectively, but YTH 34.5 contained alanine and proline. On checking the antigen binding of an intermediate form of the antibody it was found that binding was poor in comparison to the original monoclonal. By introducing mutations into two residues within the NEW first hypervariable loop, binding was restored. The second stage of humanisation involved attachment of the rat heavy chain variable domain from step one onto each of the constant domains of human IgG subclasses 1, 2, 3 and 4. Subsequent comparison of the different subclasses in complement lysis and ADCC assays indicated that the IgG1 form was the most effective at lysing target cells. The final stage was that of reassembling the IgG1 plasmid with the rat light chain CDRs cloned into the REI human light chain framework. For antibody expression in rat YO myeloma cells the engineered antibody chains were placed, in genomic context, under control of the immunoglobulin promotor/enhancer. The resulting antibody is known as Campath-1H and two patients with non-Hodgkins lymphoma (Hale et al 1988, YO source). and eight with RA (Isaacs et al 1992, CHO source) were treated in vivo. The flow diagram overleaf (Fig.1.2) represents the development strategy of the antibody from monoclonal to humanised recombinant antibody.

Figure 1.2 Development of Campath from Campath-1M monoclonal to humanised Campath-1H antibody.



1.6.4 YO Campath 1H expression system

Campath-1H expressed from YO cells was found to be of low yield (10-20 µg/ml). This was unexpected as both lymphocytes and hybridoma cells are capable of producing 200 µg/ml antibody using the immunoglobulin promotor/enhancer system (Cleveland et al 1983). The integration of DNA into the cellular genome of cells is a random event therefore, the low expression might have been a consequence of integration at a sub-optimal location. The re-cloning of the antibody genes into expression vectors bearing selectable drug resistance genes and the screening of large numbers of YO transfectants for those with high antibody expression was undertaken (Crowe et al 1992). The heavy and light chain variable regions were isolated from YO cells and placed, in genomic context with their immunoglobulin promotors, into pSV2gpt or pSV2neo plasmids, respectively. Both the immunoglobulin intron/enhancer and the human IgG1 heavy chain or kappa light chain constant domains were introduced into those plasmids as *Bam HI* DNA fragments. The resulting plasmids were designated pSVgptHuVHCAMP-HuIgG1 (heavy chain) and pSVneoHuVLCAMP-HuIgK (light chain). Electroporation of the linearised plasmids, as co-transfections, into YO myeloma cells, followed by culture in medium containing xanthine, hypoxanthine and mycophenolic acid led to the development of colonies after ten days. Selection of transfectants was performed utilising medium containing either mycophenolic acid in combination with both xanthine and hypoxanthine or, aminopterin in the presence of hypoxanthine and thymidine. Two colonies, out of 700 screened, were found to produce 40 µg/ml antibody over a 10 day period. However this was of insufficient amount to permit the treatment of large numbers of patients in clinical trials.

1.6.5 CHO Campath-1H expression system

The Campath-1H antibody used in current clinical trials is expressed in the CHO cell system using heterologous control sequences (Page and Sydenham 1991). Due to limited levels of expression in YO rat cells, the cDNA of the antibody heavy and light chains were reisolated from the YO myeloma cells and cloned into separate mammalian expression vectors under β-actin promoter/polyadenylation signals with either Simian virus (SV)-40 or metallothionein control elements directing the expression of the relevant drug selection. For the light chain cDNA the drug selection was designed around the amplifiable dihydrofolate reductase system (Simonsen and Levinson 1983) whilst the heavy chain cDNA was placed under neomycin selection (Southern and Berg 1982). Both plasmids were co-transfected into CHO cells for expression of the antibody. The resulting transfected cells were cultured in selection medium and subsequently exposed, initially to Geneticin G418 at 500 µg/ml to stabilise expression and later to varying concentrations of methotrexate (MX), an inhibitor of dihydrofolate reductase, to select for amplification of the exogenous gene copy number. When the transfectant expression levels stabilised, the cells were cloned and individual clones were found to be able to produce antibody with concentrations in the range of 200 µg/ml, a great improvement on the YO level. Initially eight patients with RA were treated with CHO Campath-1H (Isaacs et al 1992). Throughout the lifespan of each batch of antibody producing cells, the cellular DNA is monitored for Campath-IH heavy and light chain gene copy number and the DNA cleaved with restriction enzymes to detect cellular re-arrangements. Yields are assayed from each production batch and specific activity levels are assessed in standardised ADCC assays so as to be able to correct the activity of each batch for use in clinical trials. It was noted (Michelle Scott, personal communication) that yields tend to fall with continued culture and that heavy and light chain copy number fluctuated slightly. When yields become too low a fresh batch of cells are initiated.

Despite high levels of expression, CHO cells are not ideal for large scale fermentor culture. The parental cell line is an adherent cell line, whereas fermentor culture is suited to suspension cell lines. This leads to problems of clumping and settling of the cells whilst high stirring speeds in the fermentors leads to shearing of the cells. The parental CHO cells are also routinely cultured, for small scale use, in medium containing 10% (v/v) FCS. This makes the isolation of antibody expressed from large scale culture potentially difficult. The latter problem has been overcome by the development of a custom made serum-free medium (Keen 1995a), which is now used routinely for CHO clinical trial antibody production. The lack of suitable suspension host cell line has also been addressed by the use of NSO cells transfected with expression vectors bearing the Glutamine Synthetase genes (Bebbington et al 1992).

1.6.6 Recent clinical trials utilising Campath antibody

Published data recording the results from recent in vivo Campath-1H, -1G and -IM trials has been encouraging. Other than the initial affects of administration such as fevers, nausea, headaches, flu-like symptoms and rashes, the antibody has been tolerated well by the majority of patients. Trials are under way in the Royal Marsden NHS Trust in London for chronic lymphocytic and B-prolymphocytic leukaemia (Bowen et al 1997, Dyer et al 1997, Ginaldi et al 1998) plus T-cell prolymphocytic leukaemia (Pawson et al 1997); at Addenbrookes Hospital in Cambridge for multiple sclerosis (Moreau et al 1996), RA (Isaacs et al 1996a) and diffuse cutaneous scleroderma (Isaacs et al 1996b); at the Hadassah University Hospital in Jerusalem for hepatitis-associated severe aplastic anaemia (Nagler et al 1996) and for pre-transplant immunosuppression, although possible side-effects of severe migratory polyarthritis have been noted (Varadi et al 1995); at Brigham Women's Hospital in the USA for refractory RA (Weinblatt et al 1995); at the Karolinska Hospital in Stockholm for chronic lymphocytic leukaemia (Osterborg et al 1996 and 1997) and B cell lymphoma (Osterborg et al 1997). In vitro usage (Heit et al 1986, Hale and Waldmann 1994, Naparstek et al 1995) is also continuing for the T cell depletion of bone marrow samples prior to transplantation and in vivo for suppression of GVHD (Hale and Waldmann 1994).

1.7 Modifications applied to antibodies for cancer therapy

1.7.1 Radiolabelling

When utilising antibody therapy for the treatment of solid tumours, the problem of antibody penetration must be addressed. Large fragments of antibody are the least able to enter solid tissue even if the majority of the tumour has been removed by surgery. Conversely, smaller fragments, which are able to penetrate, do not initiate cytolytic effector mechanisms. Conventional radio-therapy involves either localised irradiation or whole body irradiation of the patient. The former technique may not effect outlying tumours whilst the second does not distinguish normal from diseased tissue. To enhance the action of antibody on the inner regions of tumours, anti-tumour antibodies have been coupled to radionuclides such as the β-emitter ^{90}Yttrium (^{90}Y) or the γ-emitters ^{125}Iodine (^{125}I) and^{131}Iodine (^{131}I)(Steplewski 1993). The method of coupling is critical in that the antibody should not be inactivated by the process (Awwad et al 1994). The coupling procedure may also alter the physical parameters of the antibody such as rates of decay and clearance as well as metabolism in vivo. Radiolabelled antibodies act as targeting agents which transport radionuclides specifically to the tumour site (Grossbard and Nadler 1993). The radiation emitted is of restricted pathlength and consequently limited

to localised action but, can prove toxic over several cell depths distant from the original antibody/antigen binding site. Attempts to reduce the damage caused by circulating conjugate has involved the utilisation of two step procedures in which an antibody-biotin or -streptavidin combination is given and allowed to localise to the tumour. This is followed by the administration of a radionuclide-avidin or -biotin conjugate which is captured at the site. The small size and thus better tumour penetration of biotin makes it a more suitable partner for the localisation of radionuclide to the bound antibody conjugate.

1.7.2 Linkage to chemicals

As with radiation therapy, the use of un-conjugated chemicals to treat tumours is not specific. Current chemotherapy involves the systemic application of toxic compounds which affect both normal and diseased tissue throughout the patients body. The drug is selected to be toxic only when internalised into cells. The conjugation of toxic compounds to antibody molecules, allows the directed targeting of the drug to sites of specific tumour antigen expression for internalisation of the conjugate into only tumour cells. When inside the cells, the drug is released from the antibody by proteolytic cleavage. Therefore, the choice of coupling linkage is again important (Awwad et al 1994). There must be the maximum concentration of drug molecule bound to the antibody without affecting the physical properties and yet the linkage should be readily cleaved only when inside the cell. The utilisation of naturally occurring bacterial and plant toxins, such as ricin, for this mode of therapy has also proved very efficient (Colnaghi et al 1993, Grossbard and Nadler 1993, Wallace 1994). To overcome the inherent cell binding properties of the complete toxin, the active A chain has been separated from the cell binding B chain. Subsequent conjugation of the A chain to antibody results in a very potent toxin whose action is specific on the target cell (Mansfield et al 1997a and b).

1.7.3 Pro-drug usage

The efficacy of antibody-chemical conjugates can be enhanced by the use of inert pro-drug chemical compounds. Enzyme-antibody conjugates which can activate the drug are initially targeted to the tumour by the antibody. Subsequent administration of the pro-drug leads to activation in situ and results in the localised release of comparatively large amounts of the toxic compound. This antibody-directed enzyme pro-drug therapy or ADEPT approach (Chester and Hawkins 1995) has been applied to the use of pro-drugs such as 5-fluorocytosine which is converted to the active compound 5-fluorocil by the enzyme cytosine deaminase. The major problem associated with ADEPT is that enzymes specific for the cleavage of the pro-drugs are usually not human and therefore

immunogenic (Wentworth et al 1996). To overcome this, human enzymes have been modified to be specific for the substrate (Smith et al 1997) or the antibody utilised can be generated against transition-state analogues so as to act directly on the prodrug as an abzyme and therefore replace the enzyme (Wentworth et al 1996). The latter mechanism is referred to as antibody-directed abzyme prodrug therapy or ADAPT.

1.8 Possible mechanisms of action of antibody both in vitro and in vivo

1.8.1 Antibody-dependent cellular cytotoxicity (ADCC)

ADCC is the mechanism whereby effector cells are directed to antigen expressing target cells by antigen specific IgG antibody (Fanger et al 1989). Effector cells, of both phagocytic and non-phagocytic myeloid origin as well as large granular lymphocytes (Ortaldo et al 1987), which bear FcR utilise this mechanism to bind to the complexed antibody via Fc CH2 and CH3 domains. A requirement of the mechanism is that close contact between the target and effector cells is achieved, the involvement of adhesion molecules ensures this (Robertson et al 1990, Werfel et al 1991, Jonjic et al 1992, Kushner and Cheung 1992, Voltarelli et al 1993, Majima et al 1993) and in in vitro assays there is a requirement for divalent cations (Graziano et al 1989). The process of binding activates the effector cell to release granules of perforin (Lu et al 1992, Doherty 1993, Salcedo et al 1993), granzymes (Talento et al 1992), cytotoxins and proteases (Sayers et al 1992, Doherty 1993) which puncture the cell wall and influence DNA degradation of the target cell. The receptor which is the key mediator of this event on CD56 positive NK cells is Fc RIII or CD 16 (Ritz et al 1988, Fanger et al 1989). Monocytes or CD14 positive cells, are also able to mediate ADCC activity but utilise Fc RI and Fc RII or CD 64 and CD32 respectively (Fanger et al 1989). The effector cells are defined as non-specific in that they do not rely on MHC recognition events, however for NK cells, a theory of 'missing self' has been proposed as a method of identification of the target cell (Raulet 1992). In general terms, a lack of or masked or aberrant MHC class 1 expression, is sufficient to label a cell as not-self and therefore undesirable. Cells with defects in MHC class 1 antigen processing can have sensitivity to NK lysis reversed by the transfection of TAP1/2 genes (Salcedo et al 1994). Not all MHC class 1 molecules can confer protection against NK-mediated lysis (Christiansen et al 1993, Kaufman et al 1993, Lin et al 1993, Correa et al 1994, Ciccone et al 1994). The receptor for the MHC HLA-C molecules on the NK cells has been postulated to be p58 (Moretta et al 1993). The induction of ADCC is dependent on the type and state of activity of the effector cell (Cifone et al 1993), the density of the antigen on the target and the isotype of the targeting antibody (Hale et al 1985, Ortaldo et al 1987, Miklos et al 1992, Michaelsen et

al 1993). Activity of the effector cells can be modulated by chemical mediators such as γ-interferon (IFN) (van Schie et al 1992, Martin and Edwards 1994)), α-IFN (Vuist et al 1993), granulocyte and macrophage colony stimulating factor (GM-CSF) (Bianchi et al 1989) and cytokines including interleukin (IL) 2 (Bianchi et al 1989, Naume and Espevik 1991, Chan et al 1992), IL 4 (Ting and Hargrove 1992b, Snoeck et al 1993), IL 10 (te Velde et al 1992, Gazzinelli et al 1992, D'Andrea et al 1993), natural killer cell stimulatory factor (NKSF) or IL 12 (Chehimi et al 1992, Robertson et al 1992). Inhibitors of protein synthesis have been demonstrated to augment activity (Tripathi et al 1991) as do inhibitors of protein tyrosine kinase signalling (Ting et al 1992a, O'Shea et al 1992, Borrego et al 1993, Whalen et al 1993) but not inhibitors of G binding proteins (Whalen et al 1992).

1.8.2 Complement lysis

Irrespective of effector cell activation, antibody complexed with antigen can lead to the deposition of complement component C1q on antigen bearing target cell or organism. The site for C1q binding is in the antibody CH2 domain close to residues glutamine 318, lysine 320 and lysine 322 (Duncan and Winter 1988). The levels of binding can however, be influenced by the presence or absence of the CH1 domain (Mizutani et al 1993). Antigen density is an important feature in the initiation of this mechanism as several C1q sites need to be engaged for the process to occur. Once C1q is bound, C3 convertase promotes the complement cascade by the conversion of C3 to components C3b and C3a leading ultimately to the formation of the membrane attack complex (MAC). Studies by Anton and his colleagues (Anton et al 1994) demonstrated that C3b binds directly to IgG1 at several sites in both the Fab (40% of the total) and Fc (30% of the total) regions. He suggests that this process, by the generation of conformational changes in the antibody, is one that aids the solubilisation of complexes and inhibits their precipitation. In support of these findings, antibodies engineered to reduce their Fab flexibility (Shopes 1993), continued to induce the lysis of antibody coated targets. However and in contrast, antibody isotypes which have a short or non-existent hinge region and are thus less flexible i.e. human IgG4 or mouse IgG1 were shown by Ole Brekke to be unable to fix complement (Brekke et al 1995).

1.8.3 Target cell cytostasis

Monocytes which have been activated by mechanisms such as the detection of non-self components on cells (antibody-independent) or Fc R mediated interactions with antibody complexed with antigen on cells (antibody-dependent), have the ability to influence the growth kinetics of those cells (Kobayashi et al 1992). The mediators of the cytostasis are

postulated to be soluble factors which include tumour necrosis factor (TNF)-α, transforming growth factor (TGF)-β, IL 1, oxygen radicals and nitric oxide in addition to arginine metabolism via the enzymes arginase to ornithine/urea (Mills et al 1992) or nitric oxide synthase to citrulline/NO. It was demonstrated that contact was not required for cytostasis to occur (Kobayashi et al 1992). When considering antibody mediated cytostasis, the IgG Fc RI, RII and RIII receptors are all thought to be involved. Experimentally, Levy Polat, demonstrated that by crosslinking the IgG Fc receptors on cells with goat anti-Ig $F(ab')_2$ fragments, increased TNF production was induced (Levy Polat et al 1993). Activation of monocytes can be modulated by γ-IFN (Martin et al 1994) and lipopolysaccharide (LPS)(Kobayashi et al 1992).

1.8.4 Apoptosis

Apoptosis or programmed cell death occurs as a terminal cellular response to events such as those involved with T cell clonal selection during thymic development. The mechanism is characterised by membrane blebbing of target cells, condensation of the cytoplasm of those cells, fragmentation of the cellular DNA and is very different from the appearance of necrosis. Ligands and genes, such as Fas (previously known as APO-1, Dhein et al 1992, Schulze-Osthoff et al 1994) and Bcl respectively, regulate the incidence of apoptosis and, as such, have become indicators of the mechanism. Recent evidence from Knight (Knight et al 1996) and Eischen (Eischen et al 1996) suggests that activated NK cells not only kill targets via the release of pore-forming granules but also by granule-independent mechanisms. According to Eischen's data, FcR crosslinked stimulation of NK cells induces the transcriptional up-regulation of the Fas ligand on the surface and they then interact with targets bearing Fas receptor. Antibodies which crosslink Fas (Dhein et al 1992) can induce cells to undergo apoptotic death, whilst antibodies which block the receptor inhibit the response. It has also been suggested that some anti-CD4 antibodies (Choy et al 1993) mediate monocyte induced apoptosis of CD4 cells rather than deplete by direct lysis.

1.8.5 Blockade of receptor/ligand interaction

Not all antibodies utilised for in vivo therapy are required to initiate the depletion of specific populations of cells. Prior to therapy, clinicians must define their specific antigenic target and design suitable antibody-based treatment (Chester and Hawkins 1995). For therapy with antibodies such as anti-CD4 or anti-TCR, the desired outcome might be to induce target blockade in such a way that the T cell survives but is restricted in its action and a state of anergy is induced (Steinman 1990, Kupiec-Weglinski et al 1993, Eto et al 1994, Isaacs and Waldmann 1994). This approach would be required for diseases thought to be autoimmune-related such as multiple sclerosis (Ebers 1994,

ffrench-Constant 1994, Moreau et al 1996). To achieve this action the choice of antibody isotype is critical. Antibodies which activate ADCC mechanisms via Fc R are not suitable, whereas isotypes such as IgG4 or Fc-mutated IgG1 can be utilised. Intact IgG1 antibodies can be candidates if Fab or $F(ab')_2$ fragments are constructed or as in the case of the mouse IgG2a anti-colon tumour antibody, 17-1A (Khazaeli et al 1988, Riethmuller et al 1993 and 1994), low affinity antibodies (10^{-7}) are selected. Anti-cytokine/cytokine receptor antibodies i.e. anti-TNF, anti-IL 2R (Tanaka et al 1993) and anti-IFN are administered with the objective of either engaging soluble cytokine or blocking the receptor in the body and thus reduce their ability to activate cells. Anti-adhesion molecule (ICAM-1 and LFA-1) antibodies are surmised to work by interfering with cellular interactions (Metcalfe et al 1993).

1.8.6 Opsonisation and phagocytosis

Macrophages and neutrophils are known to express complement receptors which bind to complement components deposited on target cells. Activation of the effector cells by the presence of complement induces the opsonisation and frequently phagocytosis of the target cells and their eventual destruction. Phagocytosis can be either opsonin-independent or opsonin-dependent (Krauss et al 1994) and the latter mechanism was demonstrated when both complement receptor (CR) 3 and Fc-γ RIIIB were co-expressed in usually non-phagocytic fibroblasts. The presence of both adenosine A1 (Salmon et al 1993) and histidine-rich glycoprotein in plasma (Chang et al 1992 and 1994) has been shown to enhance Fc R expression and Ig-mediated phagocytosis.

1.9 IgG Fc receptors, distribution, structure and recognition sequence

Circulatory antibodies, such as IgG1, are recognised via cell surface expressed receptors on effector cells. The receptors become activated through the action of binding antibody which initiates signalling cascades (Liebson et al 1990, Azzoni et al 1992, Wirthmueller et al 1992, Greenberg et al 1993, Agarwal et al 1993, Cone et al 1993, Stahls et al 1994) within the cell and induce typical effector cell properties. If the antibodies are themselves linked, through their Fab regions, to cell surface expressed antigen, those antigen bearing cells become the target for the effector cell action. For IgG1 there are three distinct classes of receptor, Fc RI, II and III, with all displaying polymorphism in structure and showing diversity in cell type expression.

1.9.1 Fc RI (CD 64)

This receptor is found predominantly on monocytes with lower numbers present on unstimulated macrophages. It is also displayed on activated neutrophils and eosinophils. The receptor is a member of the Ig super-family and is a 72kD glycoprotein displayed in four isoforms, two of which are soluble. The receptor possesses an extracellular region of a maximum 292 amino acids formed into two or three Ig-like domains, a 21 amino acid transmembrane region and when present, a 61 amino acid cytoplasmic region (van de Winkel and Capel 1993). It has a high affinity for monomeric IgG demonstrating a preference for subclasses IgG1 and IgG3, whilst binding IgG4 with a ten-fold lower affinity. It does not bind IgG2 at all (Woof et al 1986). The receptor synthesis is stimulated by the presence of antibody and when the receptor on monocytes is engaged by antibody complexed with cell surface antigen, it can induce the cells to exhibit ADCC activity. There has been demonstrated a requirement for the co-expression of the TCR-CD3 γ chain component for efficient signal transduction (Takai et al 1994).

The recognition sequence for Fc RI binding is located in the lower hinge region of IgG between the di-sulphide bridge and the amino-terminus of the CH2 domain in the region of amino acid residues 234-239 (Lund et al 1990, Pound et al 1993b). There is an absolute requirement for the presence of the N-linked carbohydrate on residue 297 for Fc RI binding and residue glutamine 235 appears to be critical for recognition (Woof et al 1986, Duncan et al 1988).

1.9.2 Fc RII (CD32)

Fc RII is displayed on subsets of lymphocytes (Hadley et al 1992, Mantzioris et al 1993), monocytes and macrophages, as well as cells from the polymorphonuclear lineage such as neutrophils, eosinophils and platelets (Slupsky et al 1992). It is further reported to be expressed and secreted from epidermal Langerhans cells (Astier et al 1994). The receptor is displayed as several isoforms, at least five membrane bound and one soluble. The membrane bound receptor is of 40kD and is usually composed of a 180 amino acid extracellular region formed of two Ig-like domains, a 27-29 amino acid transmembrane region and a cytoplasmic domain of variable length of between 44 and 76 amino acids (van de Winkel and Capel 1993). Complexed antibodies of subclasses IgG1 and IgG3 bind to the receptor with low affinity and in the case of platelets, activation leads to the induction of thrombosis. IgG4 binding has not been demonstrated. The site of recognition of Fc RII lies within the same region for that of Fc RI and the two possibly overlap.

1.9.3 Fc RIII (CD16)

Lymphocytes (Hadley et al 1992), macrophages, activated monocytes, neutrophils and NK cells (Ritz et al 1988) express Fc RIII with the receptor on neutrophils being linked to the cell surface via a glycosylphosphatidylinositol (GPI)-anchor. Due to extensive glycosylation the apparent molecular weight varies between 50 and 80kD. The receptor contains two Ig-like extracellular domains of 190 amino acids and, if present, a transmembrane region of 39 amino acids and a 25 amino acid cytoplasmic tail (van de Winkel and Capel 1993, Stahls et al 1994). There are two soluble, as well as membrane and GPI-anchored, isoforms. Complexes of the membrane bound receptor and a variety of di-sulphide linked dimeric subunits i.e. the γ and ζ chains of the T cell receptor (TCR)-CD3 complex (Anderson et al 1989, Jensen et al 1992, Romeo et al 1992, Moingeon et al 1992, Wirthmueller et al 1992, Biassoni et al 1993, Alevy et al 1993, Takai et al 1994), have been demonstrated to be essential for expression of the receptor and subsequent signalling events. The receptor binds complexed IgG1 and IgG3 preferentially, but also monomeric IgG with low affinity. The interaction of this receptor on NK cells with antibody complexed with cell surface bound antigen is the prime mediator of ADCC activity.

As with Fc RI and II, the recognition sequence for Fc RIII lies within the CH2 domain of the antibody. Removal of carbohydrate from the antibody leads to a subsequent loss of binding to the receptor on target cells and is suggested to be due to minor structural changes. Experiments have located the important residues close to histidine 268 (Lund et al 1990).

1.10 Glycosylphosphatidylinositol-anchored molecules

1.10.1 Types of protein anchoring

Surface associated molecules can be attached to the cell via several mechanisms the most common of which is by a transmembrane region spanning the lipid bilayer. In this arrangement there is usually an internal cytoplasmic tail of variable length through which the external molecule links to the cell signalling mechanisms and this is seen in both type one (the NH_2-terminal is extracellular) and two (the COOH-terminal is extracellular) proteins. Soluble proteins can be generated from transmembrane linked molecules by either differential splicing, early stop codons or proteolytic cleavage. Glycosyl phosphatidylinositol (GPI) attachment is an alternative form of anchoring seen in mammalian species. They were discovered in protozoan parasites in the 1980s (review Cross 1990), and involve the retention of the protein in the bilayer by the use of a lipid entity which does not span the outer cell membrane but merely sits within it. The anchor

has no internal cytoplasmic portion and therefore has high lateral mobility (Zhang et al 1991) but can signal via associated molecules (Robinson 1991, Stefanova and Horejsi 1991, Garnett et al 1993, Shenoy-Scaria et al 1993). Soluble GPI-anchored molecules can be released by cleavage with phophatidylinositol specific phospholipase C or PIPLC.

1.10.2 Amino acid sequence requirements for GPI-anchoring

Most of the evidence for consensus amino acid sequence requirements for GPI-anchors has come from work on either the decay accelerating factor (DAF) or CD55 which is associated with the prevention of damage to mammalian cells by complement (Cervoni et al 1993, Simpson and Holmes 1994), or from studies on the mouse surface thymocyte marker Thy-1 (Fasel et al 1989). DAF is a GPI-anchored molecule found on both red cells and nucleated cells and its mechanism of action is that of blocking the assembly of complement C3 convertase (Funabashi et al 1994) which eventually would lead to the damage of the cell membrane. Thy-1 is also a GPI-anchored molecule which is found on rat and mouse thymocytes and has been indicated in cell activation by crosslinking experiments (Robinson 1991). Data generated from studies on DAF indicates that there are strict amino acid sequence requirements to direct GPI-anchorage (Moran and Caras 1991a and b, Coyne et al 1993). The first necessity is that there must be a suitable small acceptor amino acid at the site of anchorage. By deletion and replacement experiments the number of suitable amino acids has been demonstrated to be limited to six, with a hierarchy within these. The most preferred amino acid is serine followed by alanine, glycine, asparagine, aspartic acid and finally cysteine in decreasing order. The second requirement is the presence of two small amino acids in positions two and three immediately downstream of the anchorage site, possibly to limit steric hindrance. Once again there are preferred amino acids in these positions with these being serine, glycine, alanine, asparagine, aspartic acid or arginine. The next requirement is that there is a spacer region of between 12-20 hydrophilic amino acids between the anchorage site and the sequences which direct the anchoring. This final region should be a stretch of at least 15-20 amino acids which are hydrophobic. In the case of DAF the GPI-anchorage information is within the last 37 COOH-residues of the coding sequence with the site of anchorage being serine 319 followed by glycine at residue 320. The spacer which is found in DAF is a 12 amino acid span rich in serine and threonines followed by a 17 amino acid hydrophobic domain which directs the anchoring. In some cases (Coyne et al 1993) it is possible to replace the natural signal region with a synthetic signal composed of three serines followed by 8-12 threonines and then 11-14 leucines and achieve GPI attachment to the first serine.

1.10.3 The structure of GPI-anchors

GPI-anchors were first described in the literature by Slein and Logan in 1963, when a bacterial enzyme, phospholipase C, was shown to be able to remove alkaline phosphatase from the surface of mammalian cells. The results were confirmed in the 1970s when purified phosphatidylinositol specific phospholipase C (PIPLC) was demonstrated to be the enzyme involved in this cleavage. However, a lack of the substrate in large quantities limited the advances in studying the anchor structure. In the 1980s it was shown by Ferguson (Ferguson et al 1995) that the variant surface glycoprotein (VSG) of the protozoan parasite *Trypanosoma (T) brucei* was also GPI-anchored. The fact that the parasites could be cultured to high density led to much of the present understanding of the basic structure. GPI anchors are added as post translational modifications to transcribed and translated nascent polypeptides which have been directed to the ER by their 5' signal sequence. The anchors are preformed in the lumen of the ER (Cross 1990, Englund 1993, Yeh et al 1994). They are added to the polypeptide by an transamidation reaction which occurs at the same time as cleavage of the polypeptide one amino acid downstream of the anchor attachment site, the process having been previously directed by the downstream cleaved sequences (Englund 1993). All anchors are composed of a basic structure to which minor modifications are added. The basic GPI core is composed of a fatty acid tail, which is the point of attachment to the cell membrane, phosphate linked to an inositol ring (Cross 1990). In turn this is linked to a glucosamine ring to which are attached three mannose residues in series. The last mannose ring is linked via another phosphate linkage to an ethanolamine residue. It is the ethanolamine residue which becomes joined to the GPI-anchoring amino acid by the transamidation reaction. Variations to the general core structure mainly fall into three categories, these being extra palmitylation of the inositol ring (Treumann et al 1995), variable glycosylation of the mannose rings including the addition of extra ethanolamine residues and the remodelling of the fatty acid tail components (Cross 1990).

1.10.4 The synthesis of GPI-anchors

Evidence for the process of GPI-anchor synthesis has been collected from various sources. Firstly, the study of a range of murine and human T cell hybridoma mutant cell lines which form a series of complementation classes (Table 1.1, copied from Yeh et al 1994). These cells have defects, in either the different steps involved in GPI synthesis pathways or lack glyco-transferases, which add GPI components to Thy-1 (Englund 1993). Secondly, from patients who suffer from an acquired X-linked defect, of varying degrees of severity, called Paroxysmal Nocturnal Haemoglobinuria or PNH (Ueda et al 1992, Takeda et al 1993, Schubert et al 1994, Bessler et al 1994). The defect results in the symptom of nocturnal lysis of red blood cells as a consequence of the reduced or absent

production of GPI-anchored molecules. GPI-anchored molecules such as DAF would normally protect the red cells from lysis (Simpson and Holmes 1994). Additionally information has been obtained from the long term study of the protozoan parasite *T. brucei* (Cross 1990).

Table1.1 GPI synthesis mutant murine T cell lines and their molecular defects. Class refers to the Thy-1 complementation status of the cells. ETN represents ethanolamine, M mannose, GlcNAc N-acetyl glucosamine and PI phosphatidylinositol.

Mutant cell line	**Class**	**Molecular defect**
BW/A, TIMI/A, S49/A	A	Transfer of GlcNAc to PI
PNH blood cells	A	Transfer of GlcNAc to PI
S1 A/B	B	Addition of M3 to M2
TIMI/C	C	Transfer of GlcNAc to PI
BW/E	E	Synthesis of mannosylated GPIs
EL4/F	F	Addition of ETN to M3
S49/H	H	Transfer of GlcNAc to PI
Ltk-	H	Transfer of GlcNAc to PI

In the early 1980s *T. brucei* parasites were shown to use GPI-anchored molecules as a host survival mechanism in which homogeneous arrays of GPI-anchored molecules were displayed sequentially on the outer parasite coat. When a host immune response to the highly immunogenic GPI molecules was raised, parasites which had developed the next phase of GPI molecules survived. The host was left with both a redundant repertoire of antibodies to the first antigen and the next generation of parasites. The pathway of GPI manufacture was determined by incorporating radiolabelled precursors into, or chemically inhibiting the synthesis of, the parasite anchors (Field and Menon 1992).

The synthesis of the GPI-anchors begins in the cytoplasmic leaflet of the ER where a UDP-glucosamine donor molecule donates a GlcNAc molecule to a PI-lipid acceptor molecule to form a GlcNAc-PI residue (Cross 1990, Englund 1993, Yeh et al 1994). From experiments using the Thy-1 T cell mutant hydridomas it has been shown that at least three enzymes are involved in this reaction and these fall into the complementation classes A, C and H (patients with the disease PNH also have a deficiency in the manufacture of GPI-anchors at this point). The complex is then de-acetylated to give GlcN-PI and both of these two intermediates are PIPLC sensitive (Yeh et al 1994). Fatty acids, usually alkylacylglycerols, are then added to the lengthening structure of GlcN-PI to generate GlcN-acyl PI which, due to its hydrophobic nature, now translocates to the luminal surface of the ER. The GPI-anchor becomes resistant to PIPLC treatment at this

point in its biosynthesis. Within the ER lumen there is another donor molecule, dolichol-P-mannose (Cross 1990, Englund 1993, Yeh et al 1994), which is common to this pathway and that of carbohydrate addition to molecules such as antibodies and it is by using this donor that the three mannose rings are sequentially added to the GlcN-acyl-PI structure. Cells that contain a defect at the complemention group B locus cannot add the third and final mannose ring and therefore have defective anchors. When all of the mannose rings have been assembled onto the precursor anchor, the addition of ethanolamine to those rings occurs (Englund 1993). Parasitic GPI-anchors, unlike mammalian anchors, do not have ethanolamine additions to all of the mannose rings (Englund 1993, Yeh et al 1994) but they do have extra glycosylation in the form of galactose and N-acetyl glucosamine residues. Cells with the complementation group F defect are deficient in the enzymes required at this step in the synthesis of GPI-anchors.

The GPI-anchors, once synthesised to this stage, are ready to be transferred to the protein. This is achieved by the simultaneous transamidation of the amino group of the ethanolamine to form an amide bond with the activated carboxyl group of the protein acceptor residue (Cross 1990, Englund 1993, Yeh et al 1994). The carboxyl-terminus of the protein, which corresponds to the residue one amino acid downstream of the anchor site, is cleaved off at the same time. At some point within the manufacture process close to this reaction the GPI-anchors are de-acylated, transferred to the cell surface and regain PIPLC sensitivity. Proteins which bear an uncleaved signal remain in the ER (Moran and Caras 1992). A final modification that can take place after the addition of the GPI anchor is that the fatty acids of the anchor can be substituted for others such that, in trypanosomes, the main component is myristic acid (Englund 1993) whilst in the mammalian placental alkaline phosphatase both steric and palmitic acids are found in equal amounts (Cross 1990).

1.10.5 Cellular distribution and potential signalling mechanism of GPI-anchored molecules

The purification of mature GPI-anchored molecules from cell membranes in Triton x-100 containing buffers results in the molecules partitioning into detergent insoluble fractions (Brown and Rose 1992, Zurzolo et al 1994, Ferguson 1994). The insolubility is seen to be acquired after the precursor molecules leave the endoplasmic reticulum and is thought to be a consequence of involvement with apical sorting mechanisms within the cell. Other molecules endowed with this property are cholesterols and glycosphingolipids (Lisanti et al 1994). Data such as this, in combination with the identification of Calveolae (Anderson 1993a and b), has led to the belief that GPI-anchored and high glycolipid content molecules cluster into distinct regions of the cell membrane (Lisanti et al 1993).

Calveolae were first described as invaginations on epithelial and endothelial cells seen on transmission electron micrographs and a key component is the 22kD protein caveolin. Caveolae were demonstrated to be distinct from clathrin coated pits, which associate with the cytoplasmic surface of the plasma membrane, when the analysis of sequenced calveolin cDNA identified putative membrane spanning domains. The internalisation of GPI-anchored molecules through mechanisms separate from clathrin coated pits was also proved (Bamezai et al 1992, Keller et al 1992) whilst protein kinase C inhibitors have been demonstrated to prevent potocytosis via internalisation of calveolae (Smart et al 1994). It has recently been proposed that GPI-anchored molecules interact with caveolin, on epithelial cells, for signalling purposes (Sargiacomo et al 1993).

In support of this, data has located calcium pumps within caveolae (Fujimoto 1993), whilst experiments (Shenoy-Scaria et al 1993 and 1994) have further demonstrated that palmitylation of the amino-terminal cysteine motif of the protein tyrosine kinases p55fgr, p56hck, p56lck and p59lyn is critical for their partition into caveolae for subsequent interaction with GPI-anchored proteins. Similar interactions are found on neutrophils (Morgan et al 1993). The cross-linking of GPI-anchored molecules on monocytes induces calcium fluxes and activation of the oxidative burst (Lund-Johansen et al 1993) whilst GPI-anchored molecules, on T cells, are regarded as accessory molecules for activation (Robinson 1991, Stefanova and Horejsi 1991, Malek et al 1994, Rowan et al 1994) although there is no evidence for a physiological role. In the absence of the T cell receptor (TCR) i.e. in TCR negative cell lines, activation fails to occur, but can be restored on transfection of the TCR gene. The loss of GPI-anchored molecules severely diminishes the cells ability to be activated by either phytohaemaglutinin (PHA) or concanavalin (Con) A (Schubert et al 1992).

1.10.6 PNH

PNH is an acquired clonal blood disorder manifested following a mutation in the phosphatidylinositol glyco-transferase (PIG) A gene (Ueda et al 1992, Takeda et al 1993, Bessler et al 1994, Schubert et al 1994). The product of the normal gene is a transferase involved in the biosynthesis of GPI-anchored molecules. The mutation commonly leads to either a frame shift in the PIG A gene or the formation of stop codons. Both scenarios culminate in a partial or total loss of GPI-anchors on short-lived stem cells of haemopoetic lineage and rarely in long-lived lymphoid lineages. Red blood cells and neutrophils isolated from PNH patients can be either normal, totally or partially deficient in GPI-anchored molecules whilst lymphocyes are not often effected. Treatment with Campath antibodies in vivo has recently been observed to occasionally induce defects referred to as PNH-like conditions within patients suffering from B cell non-Hodgkin

lymphoma (Hertenstein et al 1995) or RA (Brett et al 1996a) predominantly in lymphocytes. Three (out of twenty five) RA patients receiving Campath-1H were noted to have CDw52 negative lymphocytes. Of the lymphocytes, nearly all of the CD4 and CD8 T cells were deficient whilst 40-80% B cells were affected. The T cell defect persisted for over 20 months whilst the B cells regained expression within three months of treatment. Unlike PNH, detailed analysis of PIG A PCR products of CDw52 negative, RNA from Campath-1H treated patients performed in Glaxo-Wellcome revealed that there was no defect in the PIG A gene (Taylor et al 1997). This would suggest that the defect arose by mechanisms different from those which cause PNH. Controversially, others (Hertenstein et al 1995) have shown deletions within the PIG-A gene.

1.11 The Campath antigen (CDw52)

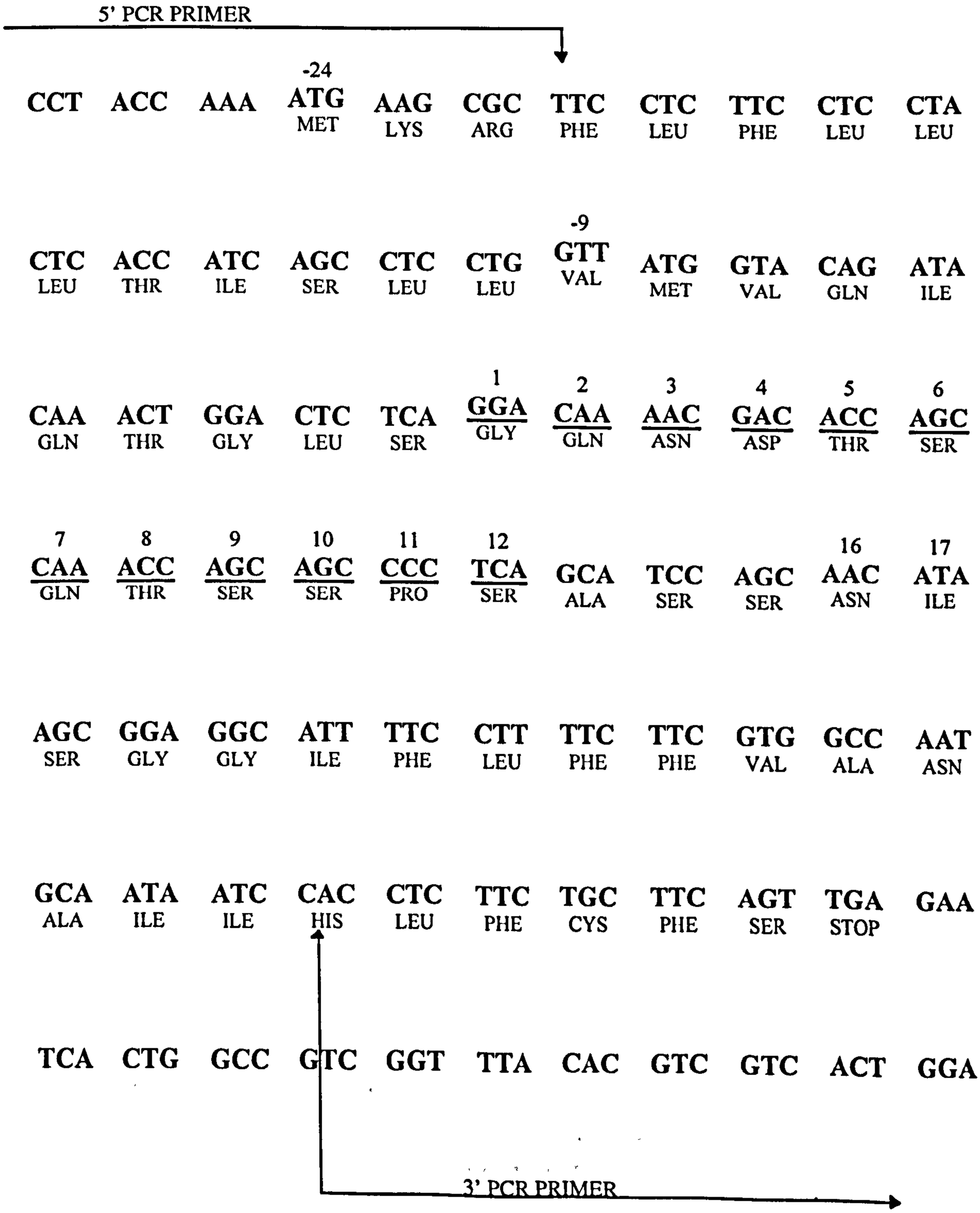
1.11.1 Sequence data

The Campath antigen (CDw52) is a cell surface molecule found on most mature lymphocytes, monocytes and sperm and is composed of a 12 amino acid mature peptide which is linked, via a GPI-anchor, to the outer membrane of the cell (Xia et al 1991 and 1993b, Hale et al 1990). The antigen is one of the smallest cell surface antigens identified up to the present time and its function is still not known. Campath antigen is however, an exceedingly good lymphocyte target for both complement (Hale et al 1983, Xia et al 1993a) and ADCC (Hale et al 1985) assays whilst Fabian (Fabian et al, 1993) demonstrated that whilst having little effect on neutrophils, both Campath-1M and -1G are also able to depress the functional activity of monocytes. The complete cDNA sequence of CDw52 was determined by the sequencing of a cDNA library (Xia et al 1991) and revealed that the 61 amino acid structure is composed of a 24 amino acid leader sequence and a 37 amino acid region consisting of the 12 amino acid mature peptide sequence and a proceeding 25 amino acid region which regulates the site of GPI-anchorage and subsequent cleavage (Fig.1.3).

The mature peptide has one N-linked glycosylation site at position three and several potential serine/threonine O-linked glycosylation sites, although there is no evidence for this type of carbohydrate being present (Xia et al 1991). The epitope for recognition, by the antibody Campath 1H, is documented to reside in the COOH-terminal portion of the antigen and has been shown to correspond to the GPI-anchor plus the last three amino acids (Xia et al 1993b) of the mature peptide. The majority of antigen moieties are, as a consequence of their GPI-anchors, sensitive to enzymatic cleavage with the enzyme PIPLC and this results in the release of the antigen from the cells and loss of reactivity to

the antibody. However, not all of the antigen is PIPLC sensitive (Treumann et al 1995) and this was shown to be primarily due to the presence of extra palmitic acid having been incorporated onto the myo-inositol ring in approximately 20% of the GPI-anchors. Therefore, PIPLC treatment of the cells only leads to a partial loss of antigen from the surface.

Figure 1.3 The published CDw52 sequence. A figure showing the position and composition of the 5' and 3' PCR primers, the amino acids in bold type, the translated products in three letter code (Maniatis et al 1982) and the major codon numbering



Two sets of sequence for CDw52 were identified in the early experiments but one sequence was thought to have been an artefact (Xia et al 1991). The two sequences, when compared, differed at two amino acid positions in the region downstream from the mature peptide in the sequences that regulates the GPI-anchorage site. Evidence presented in this thesis implies that both sequences represent forms of Campath recognised by Campath-1H antibody. The accepted cDNA sequence for Campath antigen is outlined in figure 1.3. The alternative sequence, seen in both this and the original work (Hale et al 1996, Xia et al 1991), varies at positions 16 and 17 and results in the change of these amino acids from asparagine/isoleucine to serine/methionine, a change that could generate an alternative GPI-anchoring position. Interestingly, gene products including HE5, isolated from human epidiymal tissue (Kirchhoff et al 1993), have been show to possess the same cDNA sequence as CDw52 and thus an identical protein backbone suggesting that these gene products may represent allelles. Perry (et al 1992) has also documented an similar sequence in monkey epidiymal tissue.

1.11.2 What is the evidence for the CDw52 antigenic epitope ?

Experimental evidence for the Campath antigen epitope has been derived from enzymatic cleavage of the mature protein to remove either the N-linked sugars, the O-linked sugars or the GPI-anchor (Xia et al 1991). N-glycanase treatment reduced the apparent molecular weight from 20-28kD to approximately 6kD, whilst broad specificity proteases such as pronase reduced it substantially. Neither of these treatments altered antigenicity. Alkali treatment of the antigen resulted in loss of detection by ELISA and western blot. This latter evidence was postulated to indicate that O-linked sugars were potentially involved in reactivity and other experimenters supported this theory (Valentin et al 1992). The use of the enzyme PIPLC to cleave the GPI-anchor component from CDw52 positive cells led to a reduction but not total loss in the immunofluorescent detection of those cells. Later experimentation (Xia et al 1993b) demonstrated that the isolated antigen could be separated into two fractions differing in PIPLC sensitivity. One fraction was PIPLC sensitive and the other resistant. The resistant fraction was found to bear an additional fatty acid residue on the inositol ring of the anchor which interfered with the action of the enzyme. Proteolytic digestion of the antigen showed that Campath antibody bound to a C-terminal tripeptide plus associated GPI-anchor. This was confirmed by the use of both a tri- and a twelve residue peptide corresponding to the antigen. Neither demonstrated significant binding with Campath antibody when bound directly to ELISA plates nor inhibited the binding of antibody to the native antigen, similarly bound. N-linked sugars were shown to be associated with asparagine at position three in the native antigen whilst no O-linked glycosylation was demonstrated. In 1995, the definitive

oligosaccharide structure of CDw52s N-linked sugar and GPI-anchor was published (Treumann et al 1995).

1.11.3 Distribution and biological function of CDw52

When visualised by Campath antibodies, the antigen (CDw52) is expressed on the majority of lymphocytes as well as on neutrophils, macrophages and monocytes (Hale et al 1990, Xia et al 1991 and 1993) with a density of approximately $5x10^5$ molecules per cell. Additional although refuted evidence, suggested that a similar antibody, O97, detects the antigen on erythrocytes (Valentin et al 1992). An identical cDNA transcript can be isolated from human epididymal tissue (Kirchhoff et al 1993) and a cDNA transcript similar to the CDw52 precursor has been isolated from monkey epididymal cDNA libraries (Perry et al 1992) and is demonstrated to share 85% homology with it. The potential role of CDw52 is unknown but, from its GPI-anchor, it appears similar to other GPI-anchored molecules belonging to the complement regulatory membrane protein family. This family includes membrane cofactor protein (MCP) or CD46, decay-accelerating factor (DAF) or CD55 and membrane attack complex inhibitory factor (MACIF) or CD59 and these are all GPI-anchored. These proteins are found on erythrocytes, sperm and in epididymal fluid (Simpson and Holmes 1994) and if lost from cells, as in acquired disease states such as PNH, an increased incidence of cell lysis is seen (Takeda et al 1993, Ueda et al 1992). Results presented in this thesis, in which mice intra-dermally inoculated with cells transfected with CDw52 cDNA expression plasmids developed tumours, whilst untransfected cells did not (Hale et al 1996), provides evidence that those CDw52 expressing cells may have been protected from immunological intervention in some way.

Campath antigen has been demonstrated to be an exceptionally good target for complement-mediated lysis (Hale et al 1983, Xia et al 1993a, Gilleece and Dexter 1993) and ADCC (Hale et al 1985), as well as a co-stimulatory molecule on lymphocytes (Rowan et al 1994). Perhaps it becomes a good target because the inactivation of the antigen by crosslinking enables lytic events to occur.

CHAPTER TWO: MATERIALS AND METHODS

2.1 CELLS AND MEDIUM

2.1.1 Wien 133 cells

Wien 133 cells (Nacheva et al 1987, Dyer et al 1990a), were cultured in filter sterilised Iscoves modified Dulbeccos medium (IMDM) supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS), 2 mM L-glutamine and 500 international units (IU)/ml penicillin / 500 µg/ml streptomycin. The cells were subcultured once a week by transferring 2.5 ml of the existing stock into 22.5 ml fresh medium and returning the new flask to a humid CO_2 incubator at 37°C.

2.1.2 Jurkat J6 cells

Jurkat J6 cells were cultured in filter sterilised RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FCS, 2 mM L-glutamine and 500 IU/ml penicillin / 500 µg/ml streptomycin. The cells were subcultured once a week by transferring 2.5 ml of the existing stock into 22.5 ml of fresh medium. The new flask was maintained at 37°C in a humid CO_2 incubator.

2.1.3 Chinese Hamster Ovary (CHO) dihydrofolate reductase negative ($dhfr^-$) cells

CHO ($dhfr^-$) cells were cultured in filter sterilised Dulbeccos modified Eagles medium (DMEM) supplemented with 10% (v/v) heat-inactivated FCS, 2 mM L-glutamine, non-essential amino acids, hypoxanthine, thymidine and 500 IU/ml penicillin / 500 µg/ml streptomycin. When subculturing this cell type it was necessary to loosen the adherent cell monolayer by pre-treatment with 0.02% (w/v) EDTA containing 1.25% (w/v) trypsin. The cell suspension was then diluted with an equal volume of complete medium before being centrifuged at 1500 rpm in a Sorvall RT6000B bench centrifuge to harvest the cells. Finally the cell pellet was resuspended in 10.0 ml of fresh medium to remove all residual trypsin/EDTA before taking 1.0 ml to another flask containing 24.0 ml medium. The cells were maintained at 37°C in a humid CO_2 incubator.

2.1.4 NSO myeloma cells

NSO cells were cultured in DMEM, commercially prepared to contain sodium pyruvate at 110 µg/ml but without glutamine or ferric nitrate. The medium was supplemented with 5% (v/v) heat-inactivated FCS, 1x non-essential amino acids, 1x ribonucleosides (50x

composed of 35 mg each of adenosine, guanosine, cytidine and uridine in addition to 12 mg thymidine per 100 ml), 1x glutamate and asparagine (100x composed of 600 mg of glutamic acid and 600 mg asparagine per 100 ml) plus 500 IU/ml penicillin / 500 µg/ml streptomycin. As the cells were mildly adherent, the cell monolayers were pre-treated with 0.02% (w/v) EDTA to loosen the cells prior to being washed in medium, resuspended in 10.0 ml fresh medium and then an aliquot of 1.0 ml being transferred to a fresh flask containing 24.0 ml of the same medium.

2.1.5 Transfected Jurkat cells

Jurkat cells were cultured as for the parental cell line but in addition the medium was supplemented, depending on the resident plasmid, with the selection drug of choice. In most cases the plasmid used was p321neo (Southern and Berg 1982) which confers G418 resistance and therefore the drug G418 Geneticin (Gibco) was incorporated into the medium at 500 µg/ml.

2.1.6 Transfected CHO dhfr$^-$ cells

If dhfr based plasmids were employed (Simonsen and Levinson 1983), the cells were cultured in medium which slightly differed from that used for the parental cell line and which selected for maintenance of the resident plasmid. The medium was composed of DMEM supplemented with 10% (v/v) heat-inactivated dialysed FCS, 2 mM L-glutamine, non-essential amino acids and 500 IU/ml penicillin / 500 µg/ml streptomycin. To select for increased expression of the gene of interest, methotrexate (MX) an inhibitor of dhfr, was added to the medium at an initial level of $3x10^{-8}$ M. This procedure selected for random amplification of the resident plasmid DNA associated with higher level heterologous gene expression. Once growth of the transfectants was stable at $3x10^{-8}$ M the methotrexate concentration was increased to $1x10^{-7}$ M. A repeat of several rounds of amplification led to maximal expression levels for the selected gene. The highest concentration of methotrexate employed was in the order of $1x10^{-6}$ M. If drug resistant plasmids other than dhfr based, were utilised in CHO dhfr$^-$ cells, the drug of choice was added directly to the same medium that was in use for the growth of the parental line.

2.1.7 Transfected NSO cells

For antibody production, the parental NSO cells had been electroporated in the presence of DNA encoding the heavy and light chains of the antibody of choice in plasmids containing an amplifiable glutamine synthetase cDNA (Bebbington et al 1992). This conferred resistance on the resulting cell line to the drug L-methionine sulphoximine (MSX) which is an inhibitor of glutamine synthetase. Transfected NSO cells containing these plasmids were cultured in Select medium which differed from the medium used to

culture parental cells in that it contained dialysed serum instead of normal serum and it was supplemented with various levels of MSX to select for random amplification of the foreign genes.

2.1.8 Freezing and thawing cells

Adherent and suspension cells to be frozen were taken while in a healthy state i.e. at least 95% viable. Adherent cells, were removed from the flasks by treatment with 0.02% (w/v) EDTA containing 1.25% (w/v) trypsin. The suspensions were centrifuged at 1500 rpm in a Sorvall RT6000B bench centrifuge to harvest the cells which were then resuspended at $1x10^6$/ml in their original medium containing 10% (v/v) dimethylsulphoxide (DMSO). The cells were aliquoted in 1.0 ml volumes into sterile cryotubes (Corning) and kept overnight at -70°C. The following day the frozen cells were transferred to liquid nitrogen dewars for long term storage. To recover frozen cells from liquid nitrogen, the vials of cells were taken from the dewar and, after checking that they were tightly sealed, the vial was dropped into a plastic beaker containing 37°C water. As soon as the cells had thawed the liquid was transferred to a 15.0 ml polypropylene tube containing 9.0 ml medium and the cells were washed, by centrifugation, at 1500 rpm for 5 minutes in a Sorvall RT6000B bench centrifuge. The cells were resuspended in 25.0 ml fresh medium and transferred into flasks that were placed into a CO_2 incubator at 37°C to recover.

2.1.9 Dilution cloning of transfected CHO cells

To select for highly expressing clones from pools of transfected cell lines or laboratory strains, the cells were serially diluted to 10, 3, 1 or 0.3 cells/100 µl in the relevant medium and aliquoted at 100 µl/well into 96 well flat bottomed plates, using one plate per dilution. The cells were maintained at 37°C in a CO_2 incubator for approximately two weeks and were monitored for the appearance of single colonies over that time. As colonies developed, the contents of wells containing single colonies were expanded slowly and sequentially into 48, 24, then 6 well plates and eventually up into flasks. At the 6 well stage the cells were checked for expression and a sample was frozen down in liquid nitrogen. Selected clones were maintained in 75 cm^2 flasks (Costar) during the course of the experiments and backup stocks of these clones were kept in liquid nitrogen in case of contamination.

2.1.10 Dilution cloning of NSO cells after electroporation

NSO cells ($1x10^7$ cells/ml) which had been electroporated and resuspended in 30.0 ml of myeloma culture medium were taken and 20.0 ml dispensed into the wells of 2x 96 well microtitre plates wells as 50 µl aliquots. This approximated to $1.67x10^4$ cells/well. A 10.0

ml volume of the residual cells was diluted further to give the equivalent of $4.2x10^3$ cells/50 µl and this was also dispensed into 5x 96 well microtitre plates. A 10.0 ml volume of the remaining diluted cells was diluted further to give the equivalent of $8.3x10^2$ cells/50 µl and was also dispensed into 5x 96 well plates. The cells were transferred to a 37°C CO_2 incubator for growth to occur overnight. The next day, each well was supplemented with 100 µl of the medium utilised for transfected cells and the plates replaced into the incubator to await colony development. If the plates were screened at an early stage i.e. before one month and before the medium changed colour, the numbers of single colonies per well could be assessed more accurately. Clones which produced human antibody were taken, referenced by plate, row and well (i.e. plate C7 row F well 10), and progressively expanded through 24 and 6 well plates in Celltech select medium. They were eventually expanded into 25 and 75 cm^2 flasks at which point excess cells were frozen in ampoules of 1.0 ml aliquots in liquid nitrogen. On reaching this step in the process of antibody production the selected cell lines were screened for the level of antibody production over time.

2.1.11 Calculation of specific production rates (SPR) for antibody expressed by transfected NSO cells

Following the Celltech guidelines, 75 cm^2 flasks of antibody producing cells were taken and the cells harvested by treatment with trypsin diluted in EDTA, prior to centrifugation. The cells were counted and resuspended in fresh select medium at $2.5x10^5$ viable cells/ml. Several 5.0 ml volumes of diluted cells were dispensed into 25 cm^2 flasks for overnight culture to allow the cells to adhere. The following day the medium was removed from each flask and replaced with 5.0 ml fresh, pre-warmed select medium and the flasks replaced into culture. Exactly twenty four hours later, 0.5 ml of supernatant was removed from each flask for human immunoglobulin (Ig) analysis by enzyme-linked immunosorbent assay (ELISA), whilst the remaining adherent and non-adherent cells were collected and counted. The specific production rates (SPR) were calculated as total yield of antibody in µg/24 hours/10^6 viable cells.

2.1.12 Procedure to select for the random amplification of transfected gene product

When antibody producing first round cell lines (such as the clone C7F10) had been identified and several SPR assays performed reproducibly, a strategy of selecting for random gene amplification was designed. Flasks of the cells to be screened for amplification were taken, the cells harvested and resuspended at $1x10^5$/ml in select medium. The cells were aliquoted into multiple 96 well flat bottomed plates, 100 µl/well. Twenty-four hours later, an additional 100 µl of select medium containing L-methionine-

sulphoximine (MSX) diluted to generate final concentrations of 5, 10, 15, 20 or 25 μM, was added, one concentration per one or two plates. The cells were incubated at 37°C, fed with extra relevant medium if required, until colonies reappeared (usually a period of one month). Positive colonies were designated a second series of plate, row and well numbers such as the 5 μM MSX amplification clones of C7F10, -9D4 and -10G9 (plates 9 and 10, rows D and G, wells 4 and 9).

2.1.13 Dilution cloning of amplified NSO cell lines

To guarantee clonality of the selected colonies, cultures of the clones were grown in medium containing their relevant MSX concentration and were expanded from the 96 well plates into 48, 24 and 6 well plates and on into 25 and 75 cm^2 flasks. From cultures in 75 cm^2 flasks the cells were diluted to 50, 25 and 5 cells per ml and 200 μl aliquots dispensed into 96 well flat bottomed plates and left to grow at 37°C. Colonies which appeared were given a third series title relating to plate, row and well and were gradually subcultured into increasing amounts of medium until each cell line was in a volume of 1.0 ml in a single well of 6 well plates. Aliquots of 50 μl cell supernatant were analysed for antibody content and high producing clones selected. Samples of all clones were stored frozen at -70°C.

2.1.14 Preparation of peripheral blood mononuclear cells (PBMC)

Fresh human blood (50.0 ml) from healthy randomised patients was collected from the Occupational Health Centre at Wellcome Research Laboratories, Beckenham, and defibrinated by rapid stirring with a sterile glass rod. The blood was centrifuged at 1500 rpm in a Sorvall RT6000B bench centrifuge for 5 minutes and the patients serum removed for heat inactivation (56°C for 30 minutes). The remaining cells were diluted to 80.0 ml using phosphate buffered saline (PBS) pH 7.2, and 20.0 ml aliquots were layered onto four 10.0 ml lymphoprep (Nycomed) gradients. The gradients were centrifuged at 1500 rpm for 30 minutes in a Sorvall RT6000B bench centrifuge after which the PBMC were removed from the interface above the blood cell pellets. The PBMC were resuspended in 50.0 ml PBS and kept on ice ready for further treatment.

2.1.15 Preparation of peripheral blood polymorphonuclear cells (PPMN)

Having prepared and removed the PBMC, the residual blood pellets were resuspended in 40.0 ml PBS and re-layered as 10.0 ml aliquots onto four 10.0 ml Polymorph prep (Nycomed) gradients. The gradients were spun at 1500 rpm in a Sorvall RT6000B bench centrifuge for 30 minutes and then the PPMN were harvested from the interface. The cells were washed and resuspended in RPMI 1640 medium containing 10% (v/v) FCS, 2 mM L-glutamine and 500 IU/ml penicillin / 500 μg/ml streptomycin for use in assays.

2.1.16 Preparation of peripheral blood monocytes (PBM)

PBMC prepared previously were taken and resuspended in 50.0 ml RPMI 1640 medium containing 2 mM L-glutamine, 10% (v/v) heat-inactivated autologous serum plus 500 IU/ml penicillin / 500 µg/ml streptomycin. The cells were dispensed equally into two 75 cm^2 flasks and these were placed into a humid CO_2 incubator at 37°C for three hours to allow the monocytes to adhere to the plastic. The flasks were swirled gently before the supernatants were removed to be either discarded or used to prepare other lymphocyte populations, the remaining adherent cells were rinsed several times in the same medium and then left, bathed in medium, at 37°C until required.

2.1.17 Preparation of peripheral blood lymphocytes (PBL)

Flasks in which PBM had been allowed to adhere for three hours were taken and the non-adherent cells were removed to sterile tubes. The adherent cells were washed several times with 5.0 ml aliquots of RPMI medium containing 10% (v/v) heat-inactivated FCS, 2 mM L-glutamine and 500 IU/ml penicillin / 500 µg/ml streptomycin and the washes pooled with the non-adherent cells. If the monocytes were required they were returned to the incubator bathed in medium. The non-adherent cells were pelleted and resuspended in medium to be used in further assays.

2.1.18 Preparation of peripheral blood T lymphocytes (T PBL)

PBMC prepared previously were resuspended in 50.0 ml RPMI 1640 medium containing 10% (v/v) heat-inactivated autologous serum, 2 mM L-glutamine and 500 IU/ml penicillin / 500 µg/ml streptomycin. The cells were dispensed equally into two 75 cm^2 flasks which were incubated at 37°C for three hours for the monocytes to adhere to the plastic. The non-adherent cells were subsequently removed to tubes, washed once in PBS, and then resuspended in 2.0 ml PBS to which was added a combination of 100 µl of mouse anti-human CD8 or CD4, CD16 and CD19 antibodies (Becton Dickinson), these being antisera to surface markers found on cytotoxic or helper T cells, NK cells or B cells respectively. The monocyte depleted PBMC were incubated with the antibodies at room temperature (RT) for 2 hours before being washed once in PBS and then passed slowly through a small column of fine glass beads which had previously been coated initially with myeloma protein and subsequently with rabbit anti-mouse antibody. All mouse antibody coated cells bound to the beads of the column and specific cell types could be eluted and used in further assays.

2.1.19 Preparation of mononuclear cells depleted of specific cell types

PBMC were prepared as outlined previously and were counted and diluted to $1x10^7$/ml in PBS and aliquoted into five equal samples. To each sample 100 µl of mouse antibody to a specific human cell surface marker i.e. CD3 (T cells), CD19 (B cells), CD14 (monocytes), CD56 (NK cells) or PBS was added. The cell fractions were incubated at room temperature for 1 hour and were then pipetted into sterile Petri dishes which had previously been coated with rabbit anti-mouse serum at 1 µg/ml and then washed and blocked with 1% (w/v) bovine serum albumin (BSA) in PBS. The cells were left in the Petri dishes for 1 hour at 4°C before the non-adherent cells were removed by gentle washing. The resulting cell fractions were resuspended to a concentration of $2.5x10^6$/ml based on their original cell count and used in ADCC assays. Samples of all of the aliquots were checked for their specificity by the addition of fluoroscein isothiocyanate (FITC)-labelled anti-CD3, CD14, CD19 and CD56 antibodies and by subsequent fluorescence activated cell scan (FACS) analysis.

2.2 IMMUNOASSAYS

2.2.1 FACScan (FACS) analysis and flow cytometry

2.2.1.1 Surface antigen detection

Cell lines or freshly isolated human cells were washed and resuspended in FACS buffer (5% (v/v) FCS, 0.1% sodium azide (NaN_3) in PBS) at $1x10^5$cells/ml. The cells were dispensed into 96 well U-bottomed micro-titre plates at 100 µl per well and either 10 µl FITC- or phycoerythrin (PE)- labelled antibody or 10 µl unlabelled antibody was added. The cells were incubated at 4°C for 30 minutes, to stain, before the plate was centrifuged at 1500 rpm in a Sorvall RT6000B bench centrifuge for 5 minutes. The excess liquid was removed by flicking and the cell pellets were resuspended in 100 µl wash buffer (0.1% NaN_3 in PBS) and the process repeated twice more. If the first antibody used was unlabelled, the cells were resuspended in a further 100 µl FACS buffer to which was added 10 µl FITC- or PE- labelled relevant anti-species antibody. The cells were once again incubated at 4°C for 20 minutes before being washed in wash buffer. Once the cells had been fluorescently labelled they were finally resuspended in 100 µl FACS fix buffer (1% paraformaldehyde in PBS) and either analysed on a FACScan (Becton Dickinson) immediately or left in the dark at 4°C until they could be analysed, this being no longer than three days later.

2.2.1.2 Titration of antibody/antigen binding

To compare antigen binding, samples of either concentration corrected or occasionally uncorrected test antibodies were serially diluted ten fold in FACS buffer (5% (v/v) FCS, 0.1% sodium azide (NaN_3) in PBS) and 50 µl of each dilution was placed into a well of a

U-bottomed 96 well micro-titre plate. Wien 133 cells (50 µl) diluted to $1x10^6$cells/ml in FACS buffer were dispensed into the wells and the plate incubated at 4°C for 30 minutes. The cells were washed twice in FACS buffer, utilising centrifugation at 1500 rpm in a Sorvall RT6000B bench centrifuge for 5 minutes to pellet the cells. The cells were resuspended by tapping the plate and 100 µl goat anti-human IgG-FITC labelled secondary antibody (Sigma), diluted one in 100 in FACS buffer, was added. After a further 25 minute incubation at 4°C, the cells were washed three times in FACS buffer prior to resuspension in 100 µl FACS fix buffer (1% paraformaldehyde in PBS). The results from analysis on the Becton Dickinson flow cytometer enabled the construction of binding curves utilising antibody concentration (X axis) and mean fluorescence intensity (MFI, Y axis).

2.2.2 Human IgG ELISA

To assay for antibody production from a transfected cell line, a range of dilutions of the cell supernatant was checked by ELISA for human Ig content. The assay was set up in 96 well flat bottomed plates which had been coated overnight, at 4°C, with 100 µl/well sheep anti-human IgG at a final concentration of 2 µg/ml in PBS. The coated wells were washed five times with a wash buffer (PBS containing 0.1% (v/v) Tween 20) and were then blocked overnight with 100 µl/well PBS plus 2% (w/v) BSA, again at 4°C (the assay plates could be stored at 4°C at this stage). Before the supernatant dilution's were tested, the wells were again washed three times with 100 µl/well wash buffer. A reference curve of a known antibody standard was prepared over the range 250 ng/ml to 3.9 ng/ml, using doubling dilution's in PBS containing 0.2% (w/v) BSA, 0.05% (v/v) Tween 20 (PBT), and either 100 µl volumes of the standard dilution's or the test sample dilution's were added to the assay plate in duplicate or triplicate. After 60 minutes at room temperature the plate was washed five times with the wash buffer and the wells replenished with 100 µl horseradish peroxidase (HRP) conjugated anti-human IgG antibody (Sigma) diluted one in 2000 with PBT and left at RT for a further 30 minutes. The plates were washed five times in wash buffer and the reaction developed by the addition of diluted TMB substrate (Sigma, 1 tablet dissolved in 5.0 ml water, 5.0 ml TMB buffer) 100 µl/well. The colour development was allowed to proceed until a discernible blue colour was visible and then 100 µl of 2M H_2SO_4 was added to each well. The plate was placed in a scanning spectrophotometer (Anthos) and the results monitored at 450 nm. The test sample result values were calculated from the standard curve. Those obtained from diluted samples were considered to be more accurate as they were derived from the linear region of the standard curve.

2.2.3 ADCC assay

Utilising a variation of the method devised by Ortaldo and colleagues (1987), freshly subcultured Wien 133 cells (Nacheva et al 1987) were taken and centrifuged at 1500 rpm in a Sorvall RT6000B bench centrifuge for five minutes. The cell pellet was resuspended in 200 µl sodium chromate (^{51}Cr) at 5 mCi/ml and the cells left, within lead shielding, to label at 37°C for 1 hour in a humid CO_2 incubator. The suspension was agitated gently every 20 minutes to enhance the efficiency of the reaction. Finally, the cells were washed extensively in medium and resuspended at $2x10^5$/ml, before they were aliquoted into 96 well 'U' bottomed microtitre plates at 50 µl/well.

Triplicate antibody dilutions were prepared in 200 µl medium, in fresh 96 well plates starting at the selected concentration and titering out at one in ten dilutions. A volume of 50 µl of each of the resulting dilution was transferred, in triplicate, into the plates already containing 50 µl labelled target cells. Assay controls were set up such that nine wells containing target cells were filled with 50 µl medium only. The plates were left at 37°C for 1.5 hours in a humid CO_2 incubator.

Effector (usually PBMC) cells were prepared as described elsewhere and were diluted, in medium, to a final concentration of $2.5x10^6$/ml. The effector cells were dispensed onto the antibody pre-coated labelled cells, 100 µl/well, resulting in a ratio of 25 effector cells per target cell. The effector cells were also added to one triplicate set of control wells (to monitor non-antibody mediated lysis) whilst medium alone was added to yet another set of control triplicates (to monitor spontaneous lysis). The plates were centrifuged for 5 minutes at 1500 rpm in a Sorvall RT6000B bench centrifuge and prior to being replaced at 37°C for 5 hours, 100 µl of water containing 1% (v/v) Triton x100 was added to the final set of control triplicate wells (to monitor total release). At the end of the incubation period 100 µl of medium was removed from each well and counted in a γ counter (Wallac, Wizard). The level of specific release was calculated as follows:

$$\% \text{ specific release} = \frac{(\text{mean cpm of antibody mediated release}) - (\text{mean cpm of spontaneous release})}{(\text{mean cpm of total release}) - (\text{mean cpm of spontaneous release})} \times 100$$

2.2.4 Monocyte-mediated, target cell growth inhibition assay

A method derived from that of Kobayashi et al (1992) was employed. Monocytes were harvested from cultures that were at least one week old by removing the RPMI 1640 medium overlay, adding 5.0 ml 0.02% (w/v) EDTA containing 10% (v/v) heat-inactivated FCS and replacing the flask into the 37°C incubator for 15 minutes. The cells were then released from the plastic by the addition of 5.0 ml Iscoves complete medium followed by

gentle loosening with sterile cell scrapers. The monocytes were washed twice in Iscoves complete medium, resuspended at $1x10^5$ cells/ml and dispensed into 96 well flat bottomed microtitre plates at 100 µl/well. Control plates containing 100 µl medium/well were set up at the same time. Excess monocytes were employed to check the cell phenotype by staining a small sample of each for the cell markers CD3 (T cells), CD19 (B cells), CD14 (monocytes), CD16 (Fc IgG RIII) and CD56 (NK cells) as well as a PBS control. The following day, triplicate 200 µl antibody dilutions, starting at 40 µg/ml, were prepared in Iscoves complete medium, in fresh 96 well plates. The antibodies were diluted in one in ten steps and included medium, lacking antibody, as controls. Triplicate aliquots of 50 µl of each antibody dilution were transferred into either the monocyte or control wells followed by the addition of 50 µl freshly subcultured and washed Wien 133 cells at $8x10^4$ cells/ml. The plates were returned to the 37°C humid CO_2 incubator for five days before all wells were pulsed overnight with 20 µl of low activity ^3H thymidine diluted to 0.05 µCi/ml with medium. The next day the cellular DNAs were harvested onto multiwell filter mats using a cell harvester (Skatron), the filters were dried and then counted on a Beta plate counter (Wallac) in sealed bags in the presence of 10.0 ml of scintillant. The inhibition of cell growth results were expressed as percentage of the control values and were calculated as follows.

$$\% \text{ control} = \frac{\text{(mean cpm of antibody dilution value)}}{\text{(mean cpm of control value)}} \times 100$$

2.2.5 Crosslinking assay (in the absence of effector cells)

Antibodies under investigation were initially diluted (in triplicate), to a concentration of 90 µg/ml, in 200 µl of RPMI 1640 complete medium. These were then further diluted in 96 well plates, using one in three steps, to give a range of concentrations which were then further dispensed into fresh 96 welled 'U' bottomed plates at either 50 or 100 µl/well. A second, crosslinking anti-human IgG was diluted in RPMI 1640 complete medium to a concentration of 20 µg/ml and 50 µl was aliquoted into the wells containing 50 µl of the test antibodies. Target cells, which were either Jurkat cells transfected with the gene for CDw52 or control untransfected cells, were washed twice in RPMI 1640 complete medium before they were resuspended to $1x10^5$/ml and dispensed at 100 µl/well into either the antibody alone or the crosslinked antibody samples. The plates were replaced into a 37°C humid incubator for five days before the cells were labelled overnight with 20 µl/well of filter sterilised low activity ^3H thymidine diluted to 0.05 µCi/ml with medium. The cells were harvested onto filter mats the following day using a cell harvester (Skatron) and the dried mats counted in the presence of 10.0 ml scintillant on a Beta plate

counter (Wallac). The results for both test and control target cells were calculated as follows:

$$\% \text{ of control} = \frac{\text{(mean cpm of antibody dilution value)}}{\text{(mean cpm of control value)}} \times 100$$

2.3 MOLECULAR TECHNIQUES

The majority of these techniques were derived from those described in Maniatis (et al 1982) or from kit instructions.

2.3.1 Restriction endonuclease cleavage of plasmid DNA

Plasmid DNA concentrations were routinely estimated by measuring the absorbance at the optical density (OD) of 260 nm. DNA corresponding to the required amount was dissolved in a total volume of 20-50 µl single strength digestion buffer (supplied as 10x stock for each enzyme by Boehringer Mannheim) containing the restriction enzyme or enzymes required, and incubated at 37°C for several hours. The resulting cleaved DNA was mixed with 5 µl five times concentration loading dye per 20 µl, and electrophoresed on 1% (w/v) agarose gels in single strength TBE buffer (10.5 g Tris base, 5.5 g Boric acid, 4 ml 0.5M EDTA/litre) at between 75-150 volts in a Pharmacia electrophoresis tank model G100.

2.3.2 Agarose gel loading buffer for DNA

A five times concentrated stock solution of 40% (w/v) sucrose containing 0.25% (w/v) bromophenol blue was used as a method of visualising the buffer front in all DNA agarose gels. An aliquot of 5 µl was added to each 20 µl digest at the end of the incubation time and before loading the sample onto the agarose gel.

2.3.3 Purifying cleaved DNA fragments from agarose gels

Fragments of DNA which were to be purified for further manipulations were excised, under UV light, from agarose gels with a sharp blade and the slice of gel containing the DNA was put into a small length of sterile dialysis sacking. One end of the sacking was sealed with a clip and the enclosed gel slice was immersed in 1.0 ml of single strength TBE buffer before sealing the other end. The complete bag, with gel slice, was placed into an electrophoresis tank (Pharmacia model G100) filled with single strength TBE and the current applied at 150 volts. The DNA was electroeluted from the gel into the buffer, within the sack, from where it was purified as follows. The buffer containing DNA within the dialysis sacking was applied, after mixing with 1.0 ml of sterile water, to an Elutip

column (Schlieker and Schuell) which had been previously equilibrated with a series of salt buffers (one wash with 1.0 ml of a high salt, 1.02 M NaCl, solution followed with one wash of a low salt 0.2 M NaCl, 0.02 M Tris pH 7.4, 1 mM EDTA solution). Once bound to the column the DNA was washed with 1.0 ml low salt buffer and was finally eluted with 300 µl high salt buffer. The DNA was precipitated, with 750 µl ethanol in the presence of 1 µl 10 mg/ml dextran 500 as a carrier, on dry ice for 15 minutes. After centrifugation at 14000 rpm for 10 minutes in an Eppendorf microfuge, the DNA pellet was washed in 70% ethanol and air dried. The resulting DNA was resuspended in 20 µl sterile distilled water for use.

2.3.4 Ligation of DNA fragments into molecular vectors

Purified, enzyme cleaved, DNA fragments were ligated into plasmid vectors with corresponding restriction enzyme overhangs by resuspending both together in a total volume of 16 µl sterile H_2O in 1.5 ml polypropylene Eppendorf tubes. Control reactions in which the vector or insert alone were resuspended in 16 µl of H_2O were also set up. A 2 µl aliquot of 10x T4 DNA ligase buffer (New England Biolabs) containing 1 µl 20 mM ATP was added to each reaction followed by 1 µl T4 DNA ligase (New England Biolabs). The reagents were gently mixed and quickly sedimented in an Eppendorf microfuge and were then placed in a 15°C waterbath overnight. The ligations were then either stored at -20°C or a transformation was performed.

2.3.5 Preparing competent cells

The bacterial strain DH5α is a recombinant-deficient suppressing strain of *E.coli* used for plating and growth of plasmids and cosmids and is defined in Maniatis (et al 1982) as being supE44ΔlacU169(ϕ80 lacZDM15)hsdR17recA1endA1gyrA96thi-1relA1. The data in brackets refers to an insert which permits α-complementation with the amino terminus of β-galactosidase encoded in pUC vectors (Hanahan 1983). DH5α bacterial cells were spread onto L-agar plates and cultured overnight at 37°C. A single colony was picked into 50.0 ml L-broth and cultured overnight in a shaking incubator at 37°C. A volume of 25.0 ml of the overnight culture was transferred to each of two 500.0 ml aliquots of pre-warmed sterile L-broth and cultured at 37°C until the 550 nm absorbance value reached 0.48 OD units. The bacterial cells were centrifuged at 5000 rpm in a Sorvall RC5B Superspeed centrifuge, rotor GSA, for ten minutes at 4°C and the pellets were resuspended in 400.0 ml ice-cold TFB I buffer (100 mM rubidium chloride, 50 mM manganese chloride, 30 mM potassium acetate, 10 mM calcium chloride, 15% (v/v) glycerol) pH 5.8. After cooling the cells to 4°C for 5 minutes they were recentrifuged at 5000 rpm for 5 minutes. The cells were resuspended in 40.0 ml TFB II buffer (10 mM MOPS, 10 mM rubidium chloride, 75 mM calcium chloride, 15% (v/v) glycerol) pH 6.5,

and were maintained on ice for a further 15 minutes before they were dispensed into 500 µl aliquots and snap frozen on dry ice. Competent cells made in this way were stored at -70°C until required.

2.3.6 Transformations

A 10 µl aliquot of each ligation sample was taken and mixed with 100 µl freshly thawed, ice-cold, competent DH5α cells in sterile Eppendorf tubes and the tubes were maintained on ice for a further 30 minutes. After a 1.5 minutes heat shock at 42°C, the tubes were replaced onto ice for 2 minutes and then 250 µl L-broth was added to each sample. The tubes were placed into a 37°C shaking incubator for 1 hour and the reaction mixes were then spread onto pre-warmed L-agar plates into which was incorporated 100 µg/ml ampicillin. The plates were placed, inverted, in a 37°C incubator overnight to allow bacterial growth to occur.

2.3.7 Mini-scale plasmid DNA preparations

Single colonies were picked from overnight plate cultures into 5.0 ml volumes of L-broth containing 100 µg/ml ampicillin. The cultures were grown overnight at 37°C in a shaking incubator. A volume of 3.0 ml of each culture was transferred to fresh tubes for isolation of the DNA utilising the reagents from a Wizard kit (Promega) and the remaining 2.0 ml stored at 4°C. The 3.0 ml aliquots were centrifuged at 1500 rpm in a Sorvall RT6000B bench centrifuge and the pellets resuspended in 200 µl resuspension buffer before they were transferred to 1.5 ml Eppendorf tubes. A volume of 200 µl cell lysis buffer was added to each sample and the tubes inverted several times. The reactions were stopped after 5 minutes by the addition of a further 200 µl of neutralisation buffer. The tube contents were centrifuged at 14000 rpm for 5 minutes in an Eppendorf microfuge and the supernatants transferred to fresh 1.5 ml Eppendorf tubes. The addition of 1.0 ml DNA binding resin to each sample and the application of the resin/DNA mix onto individual mini columns via a suction device led to the DNA being rescued. The column and contents were washed with 2.0 ml of the kit wash buffer and the columns centrifuged, inset into Eppendorf tubes, to remove excess buffer. The DNA was retrieved by the addition of 50 µl H_2O to each column and the eluates retrieved into fresh Eppendorf tubes by centrifugation. The DNA was either stored at -20°C until required or analysed by either restriction enzyme analysis or sequencing.

2.3.8 Maxi-scale plasmid DNA preparations

A small 100 µl sample (either a residual mini-scale bacterial culture or a glycerol stock of a culture) was used as a seed for a large scale plasmid DNA preparation. The plasmid

harbouring bacterial culture was grown overnight, shaking at 37°C, in 500.0 ml L-broth into which was incorporated 100 µg/ml ampicillin. The following day each culture was centrifuged in sterile centrifuge pots at 5000 rpm and at 4°C in a Sorvall RC5B Superspeed centrifuge, rotor GSA, having previously removed a 2.5 ml aliquot as a stock sample. The 2.5 ml sample was mixed with 2.5 ml glycerol and stored frozen at -20°C as a future starter culture. The remaining bacterial pellet was resuspended, in the centrifuge pot, using a total of 20.0 ml of a lysozyme mix (0.25 g lysozyme, 0.45 g D-glucose, 1.5 ml Tris-HCl pH 8.0 and 1.0 ml 0.5M EDTA per 50.0 ml water) and left at RT for five minutes. At the end of the incubation period 40.0 ml of a SDS/NaOH buffer was added (4.0 ml 5M NaOH, 10.0 ml 10% (w/v) SDS per 100.0 ml water) and the mix was shaken gently and maintained on ice for fifteen minutes. Finally, 20.0 ml of a lysis buffer (3M potassium acetate which was 5M with respect to acetic acid) was added and the mix shaken and returned to ice for a further five minutes. The resulting plasmid lysate was centrifuged at 8000 rpm in a Sorvall RC5B Superspeed centrifuge, rotor GSA, to remove the debris. The supernatant was filtered through gauze into a fresh centrifuge pot and the DNA precipitated with 48.0 ml iso-propanol, followed by centrifugation, in a Sorvall RC5B Superspeed centrifuge, at 5000 rpm. The DNA pellet was air-dried before preparing a Caesium chloride ($CsCl_2$) gradient. For the gradient, the DNA was resuspended in 9.5 ml TE pH 8.0 (10 mM Tris-HCL pH 8.0, 1 mM EDTA) to which was added 10.5 g $CsCl_2$. The $CsCl_2$ was agitated until dissolved and then 660 µl 1M Tris-HCL pH 8.0, 120 µl 0.5M EDTA and 660 µl ethidium bromide 10 mg/ml was added. The sample was loaded, using a syringe with an attached 19G needle, into a 10.0 ml heat-sealable tube which was sealed and centrifuged at 45000 rpm for two days using a 70.1 Ti rotor in a L8-55 ultra-centrifuge (Beckman).

2.3.9 Recovering plasmid DNA from Caesium chloride gradients

The heat-sealable tube containing the plasmid DNA was recovered from the ultra-centrifuge. The tube was clamped upright over a UV light source and above a beaker, and the top of the tube was pierced with a sterile needle. The UV light source was illuminated and the DNA band visualised via the incorporated ethidium bromide. A wide bore (19G) needle attached to a 2.0 ml syringe was used to puncture the tube at a point adjacent to the plasmid DNA layer and the DNA removed by suction into the syringe. The DNA was expelled into a sterile polypropylene tube and the waste gradient materials were allowed to drain into the beaker for disposal. The ethidium bromide was dissociated from the DNA by vortexing the sample with an equal volume of N-butanol and removing the upper colourless layer to a sterile Corex tube. The DNA was diluted with an equal volume of sterile distilled water and precipitated with 2.5 volumes of ethanol on dry ice for 15 minutes. After centrifugation at 8000 rpm at 4°C in a Sorvall RC5B Superspeed

centrifuge, rotor SS-34, the pellet was resuspended in 70% ethanol and re-centrifuged to precipitate the DNA. Finally the DNA was dissolved in 400 µl sterile distilled water and the OD value was recorded at 260 nm. The DNA sample was then stored at -20°C until required.

2.3.10 Transfection of DNA into mammalian cells other than NSO

Adherent cells to be transfected were aliquoted (10.0 ml) into 10 cm tissue culture grade Petri dishes at 1-5x10^5 cells/ml medium and incubated overnight at 37°C in a CO_2 incubator. Before transfection the cells were washed several times with serum-free medium and were maintained in 5.0 ml serum-free medium in the 37°C CO_2 incubator until required. Suspension cultures, prior to transfection were taken from their stock flask and centrifuged in a Sorvall RT6000B bench centrifuge at 1500 rpm to pellet the cells. The cells were washed several times in serum-free medium before they were counted, adjusted to 0.4-2x10^6/ml and 5.0 ml aliquoted into tissue culture plates. The DNA to be transfected was diluted with 0.3 M NaCl to a concentration of between 1-10 µg in a final volume of 250 µl and mixed with an equal quantity of Transfectam (Promega) solution at a concentration of 40 µg/ml. The resulting 500 µl DNA/Transfectam mix was dripped evenly onto the plate of cells to be transfected, the plate was gently swirled to mix and placed at 37°C for five hours. At the end of the incubation period, 5.0 ml of medium containing 10% (v/v) FCS was added to each plate and the cells were then incubated at 37°C for 2 days. After this period the cells were sub-divided, at a range of dilutions, into medium containing the selection drug of choice depending on the original plasmid vector used. Over the following two weeks the cells were monitored for the outgrowth of transfected cell colonies which were then checked for expression by various methods.

2.3.11 Electroporation of the final Campath-1H plasmid into NSO cells

Following Celltech guidelines, 40 µg of the plasmid pEE6H12L#4 was taken and linearised overnight at 37°C with the restriction enzyme *Sal I*. The cleaved DNA was vortexed with an equal volume of phenol/chloroform mixed 1:1, centrifuged in an Eppendorf microfuge at 14000 rpm and the resulting aqueous layer treated with one volume of chloroform and recentrifuged. The upper aqueous layer was precipitated with 2.5 volumes ethanol in the presence of 150 mM NaCl. Post precipitation at -80°C for 30 minutes, the DNA was washed in 70% (v/v) ethanol using an Eppendorf microfuge at 14000 rpm and the pellet air-dried before resuspension in 50 µl sterile distilled water. NSO cells which were to be electroporated and had been subcultured the previous day, were treated with 0.02% (w/v) EDTA to release all adherent plus non-adherent cells and the cell suspensions were pooled and pelleted by centrifugation at 1500 rpm in a Sorvall RT6000B. After washing and resuspension in ice-cold PBS, the cells were counted and

adjusted to $1x10^7$/ml before 1.0 ml aliquots were incubated in cuvettes on ice with either 50 µl experimental DNA (equivalent to 40 µg), 50 µl control vector DNA or 50 µl PBS for five minutes followed by electroporation with a capacitance of 2 pulses of 3 µF, using a Biorad Gene pulser. The cuvettes were replaced on ice for two minutes prior to the cells being rinsed out into 30.0 ml of pre-warmed medium.

2.3.12 Recovery of cellular DNA from cell cultures

DNA transfected or untransfected parental cells were harvested from tissue culture, utilising EDTA and trypsin if necessary, and the cell pellets were washed extensively in PBS by centrifugation in a Sorvall RT6000B bench centrifuge at 1500 rpm. Prior to the final spin, the cells were counted using trypan blue exclusion and after centrifugation were resuspended in TE (Tris 10 mM, EDTA 1 mM) pH 8.0, containing 100 µg/ml proteinase K (BDH), at 10^8 cells/ml. Once the cells had dissolved, a small volume of 10% (w/v) SDS was added to a final concentration of 0.5% SDS and the tube gently inverted to mix the contents. The cell mix was incubated in a 37°C waterbath for three hours before sodium chloride was added to a final concentration of 150 mM. The DNA was isolated by two treatments with an equal volume of Tris buffered phenol pH 7.0-8.0 in which the upper aqueous phase was retained after each centrifugation. This step was followed by a subsequent treatment of the aqueous layer with an equal volume of phenol/chloroform mixed in the ratio of 1:1. The resulting aqueous layer was precipitated overnight at -20°C in the presence of 2.5 volumes ice-cold 100% (v/v) ethanol. The following day the DNA pellet was washed once with 70% (v/v) ethanol and the pellet air-dried. After resuspension of 10^8 cells in 0.5 ml TE pH 8.0, RNAase A was added to a final concentration of 20 µg/ml and the mix placed at 37°C, in a waterbath, for two hours. The RNA-free DNA was adjusted to 0.2 M sodium chloride/0.1% (w/v) SDS, and an aliquot of proteinase K was added to give a concentration of 100 µg/ml and the mixture placed at 37°C for a further two hours. The DNA was isolated utilising one phenol treatment followed by one phenol/chloroform treatment as previously described. The aqueous layer was mixed with 2.5 volumes of 100% (v/v) ethanol and placed at -20°C overnight to precipitate the DNA. To perform assays with the DNA, the DNA pellet was washed once in 70% (v/v) ethanol, air-dried and subsequently redissolved in a small volume of sterile distilled water. An OD reading at 260 nm was used to assess the amount of DNA present.

2.3.13 Southern blotting of cellular DNA

2.3.13.1 Preparation of samples for electrophoresis

To assess gene copy number and the presence of desired DNA gene fragments, purified DNA was cleaved utilising restriction enzymes which were known to cleave at certain

DNA sequences and yield restriction fragments of predicted sizes. The DNA to be cleaved was prediluted with a single strength buffer, which was supplied commercially as a 10x stock (Gibco or Boehringer Mannheim), several hours prior to the addition of the required enzyme or enzymes, to aid dissolution of the high molecular weight DNA. The enzyme/enzymes, of choice, was incorporated into the DNA/buffer mix such that it constituted no more than 10% (v/v) of the total volume and the solution placed at 37°C overnight. The following day, the cleaved DNA was precipitated with 100% (v/v) ethanol in the presence of 150 mM sodium chloride, at -80°C on solid CO_2, for 30 minutes, washed once in 70% (v/v) ethanol and air-dried. The final cleaved DNA was resuspended in a small volume of sterile distilled water and the DNA content assessed by OD. A small amount, 0.5-1.0 µg, of the cleaved DNA was checked for complete digestion by electrophoresis on a 1% (w/v) agarose gel. For the actual experimental gel, 1.0-10.0 µg of cleaved DNA was loaded into the wells of a 250.0 ml 1% (w/v) agarose gel alongside molecular weight markers and control samples which were either parental cellular DNA as negative control or parental DNA spiked with known amounts of the expected DNA fragments to act as gene copy number positive controls. The gel was electrophoresed at 30 volts overnight and photographed before transfer onto either nitro-cellulose or nylon sheets.

2.3.13.2 Blotting of the electrophoresis products from gel to paper

To blot onto paper, the gel was first soaked in 250.0 ml 0.25 M HCl for fifteen minutes to depurinate the DNA and ensure even transfer of all sizes of fragments. After rinsing in distilled water, the gel was further treated with two, thirty minute washes in 250.0 ml 500 mM NaOH, 1 M NaCl to denature the DNA and finally the gel was neutralised with two, thirty minute washes in 250.0 ml 500 mM Tris pH 7.4, 2.5 M NaCl. Preferentially the gel was blotted onto nylon Hybond-N sheets (Amersham) utilising the method outlined by Southern (1975). This process involved transfer of the DNA, by capillary action via 10x SSC buffer (0.15 M Na citrate, 1.5 M NaCl), from the gel onto the paper of choice by the use of a thick filter paper wick. The nylon paper retaining the transferred DNA was air-dried, wrapped in Saran wrap and briefly exposed to ultra-violet light, in a Stratagene cross-linker, to adhere the DNA to the paper.

2.3.13.3 Probing Southern blots

Nitro-cellulose filters containing UV cross-linked DNA were placed into Techne glass hybridisation tubes with 50.0 ml, 65°C pre-heated pre-hydridisation solution (6x SSC, 5x Denhardts solution, 0.5% (w/v) SDS and 100 µg/ml single stranded calf intestinal DNA) and were rotated at 65°C for at least one hour. The pre-hybridisation solution was then removed and replaced with a fresh volume of 25.0 ml pre-heated solution containing an

aliquot, equivalent to $5x10^6$ counts per minute (cpm), of labelled probe which had been freshly heated to 95°C for 5 minutes. The filters were rotated in the hybridisation solution at 65°C overnight. The filters were washed using two 15 minute rinses in 2x SSC at 65°C, followed by two thirty minute washes in 2x SSC containing 0.1% (w/v) SDS at 65°C and finally two 10 minute rinses in 0.1x SSC at 65°C. The filters were then air-dried and placed into a photographic cassette overlaid with photographic film. After an overnight exposure at -80°C the film was developed and, if necessary, a longer exposure was set up.

2.3.14 Labelling of DNA fragments for use as probes and markers

2.3.14.1 Nick translation of DNA probes

DNA fragments, between 250 and 1500 bp long, were isolated as mentioned previously in section 2.3.3 and the DNA resuspended in a small volume of distilled water. The DNA concentration was calculated spectrophotometrically and a volume equivalent to 1 µg was placed in a sterile Eppendorf tube and the volume made up to 9 µl with water. To label, a 4 µl volume of dNTP mix (minus dATP), followed by 5 µl α^{32}P dATP and 2 µl DNA polymerase 1 (all from an Amersham Nick translation kit) was added to the DNA, mixed and the tube placed in a 15°C waterbath for 2 hours. To stop the reaction, 30 µl of water and 3.3 µl 5M NaOH were added and the reaction placed at room temperature for 10 minutes. On the addition of a further 46.7 µl distilled water the probe was purified on a small column of G.50 Sepharose. The labelled DNA was eluted and 5 µl counted in a Beta counter (Wallac). A volume equivalent to $5x10^6$ cpm was used to probe Southern blots.

2.3.14.2 Random priming of DNA probes

To achieve labelling of higher specific activity than that possible with the nick translation method, a random priming kit (Prime-it) from Stratagene was employed. A volume of purified DNA solution, equivalent to 1 µg, was adjusted to 24 µl, with water, and 10 µl of random nonanucleotide primers (27 OD units/ml) was added. After heating to 95°C for 5 minutes and cooling to room temperature, 10 µl 5x buffer (0.1M dNTPs minus dATP), 5 µl α^{32}P dATP and 1 µl T7 DNA polymerase (2 U/µl) was added and the reaction allowed to continue at 37°C for 10 minutes before the addition of 2 µl stop mix (100 µl 0.5 M EDTA pH 8.0). The probes were purified by passage through G.50 columns and a 5 µl sample of the eluted product counted on a Beta counter (Wallac). A volume equivalent to $5x10^6$ cpm was used to probe blots.

2.3.14.3 End labelling of markers for Southern blots

To assist the size determination of labelled fragments on Southern blots, it was advantageous to include radio-labelled markers on each gel. To achieve labelling of markers which were of varying fragment length, the method of end labelling was employed. A 15 µl volume of commercially made Lambda DNA (Boehringer Mannheim), enzymatically digested with both *Eco RI* and *Hind III* (Boehringer Mannheim), was mixed with 3 µl distilled water, 3 µl 10x Klenow buffer (0.5 M Tris-HCl pH 7.6, 0.1 M $MgCl_2$), 3 µl dNTP mix minus dATP (20 mM each), 5 µl α^{32}P dATP and 1 µl Klenow polymerase I. The reaction was allowed to proceed at room temperature for 20 minutes followed by 20 minutes at 37°C and finally, 10 minutes at 65°C. The probe volume was adjusted to 100 µl with distilled water and the labelled DNA purified on a column of G.50 Sepharose. After counting a 5 µl sample in a Wallac Beta counter, a volume equivalent to $5x10^6$ cpm was incorporated into each experimental gel run in addition to unlabelled markers.

2.3.14.4 Campath antigen RNA nuclear run-on probes

To probe cellular RNA extracts for Campath antigen RNA levels, it was necessary to radioactively label the RNA transcripts from a Bluescript KS^+ vector based plasmid (Stratagene), in which the gene encoding the antigen CDw52 was cloned as an *Hind III / Eco RI* insert. The vector incorporates the T3 and T7 promotors either side of the multiple cloning site to aid in vitro transcription in either orientation. The plasmid was linearised with either of the restriction enzymes *Xba I* (forward probe) or *Asp 718* (reverse probe) to ensure transcripts of known length. The probes were transcribed, employing reagents from a Promega RNA protection kit in either forward (T3 promotor) or reverse (T7 promotor) orientation, by mixing 0.1 µg of linearised plasmid with 3.5 µl distilled water, 4 µl 5x buffer, 2 µl 100 mM dithiothreitol (DTT), 1 µl RNAase-inhibitor, 4 µl dNTP mix minus dCTP, 1 µl dCTP, 2.5 µl α^{32}P dCTP and 1 µl of the relevant promotor polymerase. After gentle mixing and centrifugation, the reaction was incubated at 37°C for 1 hour proceeded by the addition of 1 µl RNAase and 1 µl RQ DNAase and further incubation at 37°C for 15 minutes. The reaction was terminated by adding 25 µl TE (10 mM Tris-HCl, 1 mM EDTA pH 8.0) followed by purification of the probes via centrifugation through pre-equilibrated microspin columns (Pharmacia). A 1 µl sample was counted in a Beta counter (Wallac) and a volume equivalent to 10^5 cpm used in each assay.

2.3.15 Nuclear run-on assays for the detection of Campath antigen (CDw52)

To assay for Campath RNA transcripts, cells were harvested by centrifugation at 1500 rpm in a Sorvall RT6000B bench centrifuge, washed in PBS three times and resuspended

at 10^7/ml in the lysis buffer supplied in a kit from the company Upstate Biotechnology (USB). The cell pellets and buffer were mixed by vortexing, left on ice for 10 minutes to allow lysis to proceed and the lysates frozen at -80°C until required. On thawing, 50 µl aliquots of each lysate were mixed with 10^5 cpm of either forward (control) or reverse (test) orientation nuclear run-on labelled probe and the reaction tubes placed at 37°C overnight to allow annealing. Once the annealing reaction had occurred, 500 µl of an RNAase mix (450 µl water, 50 µl 10x buffer plus 2 µl RNAase) was added to each tube and the tubes were gently inverted and placed at 37°C for 30 minutes to digest any remaining free probe. This was followed by the addition of 20 µl 10% (w/v) SDS and 20 µl protease also from a Promega RNA protection kit. The tubes were vortexed, quickly centrifuged and returned to 37°C for 30 minutes prior to the addition of 500 µl iso-propanol. The precipitated labelled fragments were pelletted by centrifugation in an Eppendorf microfuge at 14000 rpm, washed in 180 µl 70% (v/v) ethanol and the pellets air-dried. Each pellet was resuspended in 10 µl sequencing dye from a Sequenase 2 kit (USB), heated to 95°C for 5 minutes and 5 µl loaded onto a 75.0 ml prewarmed 6% sequencing gel alongside 5 µl labelled pΦ174 / *Hinf* size markers and 1-5 µl undigested probe. After a 2 hour electrophoresis run at 40 mA the gel was transferred to Whatman 3MM paper, dried at 70°C and placed into an autoradiograph cassette with Kodak film. The film was developed the next day.

2.3.16 Purification and isolation of cellular RNA

Cells were harvested from flasks, pelletted by centrifugation in a Sorvall RT6000B bench centrifuge, washed in PBS three times and counted. The cells were resuspended to 10^7/ml in an aliquot of stock solution (250 g guanidinium iso-thiocyanate, (Fluka/Sigma) dissolved, at 65°C, in 293.0 ml water; 17.6 ml 0.75M sodium citrate pH 7.0; 26.4 ml 10% (v/v) sarcosyl), 36 µl of β-mercaptoethanol was added to each 5.0 ml aliquot immediately prior to addition to the cell pellet (Chomczynski and Sacchi 1987). A 1/10th volume of 2M sodium acetate, an equal volume of phenol: water (1:1) and 1/5th volume chloroform: isoamyl alcohol (24:1) was added sequentially and the reaction tubes were shaken vigorously for 10 seconds and placed on ice for 15 minutes. Following centrifugation at 14000 rpm, in an Eppendorf microfuge at 4°C, each upper aqueous layer was removed to a fresh tube, an equal volume of iso-propanol was added and the tube was placed at -20°C for 1 hour. A further centrifugation at 14000 rpm, at 4°C for 20 minutes was followed by the resuspension of the resulting pellet in the guanidinium iso-thiocyanate plus β-mercaptoethanol solution (300 µl/ml) and an equal volume of isopropanol was added. After at least 1 hour at -20°C the solution was centrifuged for 10 minutes, 14000 rpm at 4°C, washed in 75% (v/v) ethanol, recentrifuged and the pellet air-dried. When required,

the RNA pellet was redissolved in 50 µl sterile distilled water which had previously been treated with di-ethyl pyrocarbonate (DEPC).

2.3.17 The preparation of messenger RNA (m-RNA)

Total RNA was assessed for RNA content spectrophotometrically at 260 nm and a volume equivalent to 75 µg was adjusted to 100 µl with DEPC treated distilled water. The RNA solution was heated to 65°C for 2 minutes and added to 1 mg of pre-washed magnetic Dynabeads from a m-RNA purification kit (Dynal). Following gentle mixing and hydridisation of the RNA to the beads for 3-5 minutes, the reaction tube was placed within the Dynal MPC-E-1 magnet for 30 seconds and whilst in situ, the excess supernatant removed. The beads, plus bound m-RNA, were washed twice with 200 µl of washing buffer as per kit instructions. The application of a small volume of elution buffer (5 µl) followed by heating the reaction mix to 65°C for 2 minutes and subsequent use of the magnet, enabled the eluted m-RNA to be removed in solution. The m-RNA was stored frozen at -70°C until required.

2.3.18 The preparation of cDNA from m-RNA

2.3.18.1 1st strand synthesis of cDNA

Reagents from a Superscript kit (Gibco/Life Technologies) were employed to convert m-RNA to first strand cDNA. A volume of m-RNA equivalent to 5 µg, plus 2 µl of 5 µg/µl *Not 1* primer-adaptors, was diluted to 10 µl with DEPC treated water. The mixture was heated to 70°C for 10 minutes and quickly cooled on ice. The tube contents were briefly centrifuged in a microfuge prior to the addition of 4 µl 5x first strand buffer, 2 µl 0.1M DTT, 1 µl 10 mM dNTP mix and 1 µl α-^{32}P dCTP (Amersham 1µCi/µl). Once mixed, the reaction was allowed to equilibrate at 37°C for 2 minutes prior to the addition of 2 µl (400 units or U) Superscript Reverse Transcriptase. A further incubation at 37°C for one hour followed. The reaction was terminated by placing the tube on ice and to calculate first strand dCTP specific activity and cDNA yield, a 2 µl sample was removed, mixed with 43 µl 20 mM EDTA plus 5 µl yeast t-RNA for subsequent dotting onto duplicate glass fibre filters. The remaining 18 µl was taken directly onto second strand synthesis.

2.3.18.2 2nd strand synthesis of cDNA

On ice, 18 µl first strand cDNA was mixed with 93 µl DEPC treated water, 30 µl 5x second strand buffer, 3 µl 10 mM dNTP mix (α^{32}P dCTP is optional for this step), 1 µl 10 unit/µl *E. coli* DNA ligase, 4 µl 10 unit/µl *E. coli* DNA polymerase and 1 µl 2 unit/µl *E. coli* RNAase H. The tube contents were mixed and incubated at 16°C for 2 hours prior to the addition of 2 µl (10 U) T4 DNA ligase. The reaction was incubated for a further 5 minutes at 16°C before termination with 10 µl 0.5M EDTA. If radioactive dCTP had

been incorporated in the assay, a 10 µl sample was removed and mixed with 35 µl 20 mM EDTA plus 5 µl yeast t-RNA for second strand yield calculations. The remaining reaction mix was vortexed thoroughly with 150 µl phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged in an Eppendorf microfuge for 5 minutes at 14000 rpm. The upper aqueous phase was removed to a fresh tube containing 70 µl 7.5M ammonium acetate and 500 µl -20°C absolute ethanol was added and the tube immediately centrifuged for 20 minutes at 14000 rpm. The supernatant was carefully discarded and the pellet washed with 180 µl 70% (v/v) ethanol prior to drying at 37°C for 10 minutes. The cDNA was resuspended in sterile distilled water and stored at -70°C.

2.3.19 Polymerase chain reaction (PCR)

Reagents from a Perkin Elmer GeneAmp PCR kit were employed for routine amplification assays (Saiki et al 1988). Initially an overlay of 100 µl of light mineral oil (Sigma) was added to each 0.5 ml sterile Eppendorf tube followed by the individual reagents in the following order, 61.5 µl sterile water, 10 µl 10x amplification buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$ and 0.01% (w/v) gelatine), 8 µl of a blend of dNTP's (mixed 1:1:1:1, 200 µM each final concentration), 0.5 µl Taq polymerase (500 units/100 µl, Perkin Elmer Cetus), 5 µl each of 0.2 µg/µl forward and reverse 20-30 mer oligonucleotide primers and finally either 100 ng of plasmid DNA, cellular DNA or cDNA in a 10 µl volume. The specific primers for each target were custom made by Hugh Spence in the Department of Molecular Biology, Wellcome Research Laboratories, Beckenham. Controls for each assay routinely included known positive controls (if available), plus sample tubes containing mixes minus either target DNA or primers. In the situation in which several samples shared common components, these were made up as a premix from which aliquots were removed into separate sample tubes prior to the addition of DNA. The components were vortexed and gently sedimented by centrifugation, the tubes placed into a Hybaid intelligent thermocycler block and a pre-programmed series of one 1.5 minute 94°C strand separation step followed by thirty cycles of 37°C for two minutes, 72°C for three minutes, 94°C for one minute, executed. A final cycle of 37°C for two minutes, 72°C for ten minutes prior to cooling, completed each amplification experiment. PCR products were isolated from the overlay of oil by the addition of 100 µl of chloroform to, and the vortexing and centrifugation of, each tube and the removal of the upper aqueous layer to a fresh tube for storage at -20°C. Confirmation of PCR product was shown by the electrophoresis of a 10 µl aliquot of the aqueous layer (mixed with 5 µl DNA loading dye) on a 50.0 ml 3% (w/v) GTG agarose (FMC): 1% (w/v) agarose gel.

2.3.20 Sequencing of cloned DNA inserts

Aliquots of 1-5 µg plasmid preparation were placed into a sterile 0.5 ml tube and the volume adjusted to 18 µl with distilled water. A 2 µl amount of fresh 2M NaOH/2 mM EDTA was added and the tube contents mixed and placed at 65°C for 15 minutes prior to the addition of 2 µl 3M NaOAc and 50 µl 100% ethanol. The tube was placed on dry ice for 30 minutes to allow precipitation of the denatured DNA. The resulting pellet was washed in 180 µl 70% ethanol by centrifugation in an Eppendorf microfuge, 14000 rpm for 5 minutes and air-dried. The pellet was resuspended in 6 µl of distilled water and utilising reagents from a USB Sequenase 2 kit (Tabor and Richardson 1987 and 1989), mixed with 4 µl annealing mix (1 µl relevant sequencing primer, 2 µl 5x buffer, 1 µl water). The tube contents were sedimented by quick centrifugation, heated to 65°C for 10 minutes and annealed by slow cooling to room temperature. After cooling, a 5.5 µl volume of labelling mix (2 µl of 1 in 5 diluted dNTP's, 2 µl 1 in 8 diluted Sequenase T7 DNA polymerase, 1 µl 0.1M DTT and 0.5 µl ^{35}S dATP) was added to the sample on ice and the tube transferred to 37°C for 15 minutes. To permit strand extension and termination, 3.5 µl aliquots of the mix were transferred into four fresh tubes, each of which contained 2.5 µl of either pre-heated ddATP, ddCTP, ddGTP or ddTTP respectively and the tubes replaced at 37°C for 10 minutes. The reactions were stopped by the addition of 4 µl dye mix and were often stored frozen, at this stage, until required. After heating to 95°C for 2 minutes, 4 µl samples of the sequencing reactions were electrophoresed on a prewarmed 8% sequencing gel which had been assembled the previous day. The electrophoresis conditions were 40 mAmp (mA) for 1.5 hours. The reloading and further electrophoresis of additional 4 µl aliquots of the same samples was used if longer stretches of sequence were required. Transfer of the gel onto 3MM paper (Whatman), which was subsequently dried and exposed to Kodak autoradiographic film, led to the visualisation of the sequence patterns.

2.3.21 The preparation of sequencing gels

A volume of 75.0 ml urea/SDS gel mix 8 from Gibco/Life Technologies was mixed with 450 µl 1% (w/v) ammonium persulphate. This was then gradually pipetted into a pre-assembled sequencing mould composed of two large glass plates, separated by two 0.4 mm spacers, sandwiched together and taped on both sides and the bottom with waterproof sequencing tape (Gibco). Whilst still liquid, a 0.4 mm 48-toothed sharkstooth comb was inserted into the top of the gel and the whole assembly rested almost horizontal to solidify. When set, the top of the gel assembly was wrapped in Saran cling wrap and the gel stored overnight at 4°C. To prewarm, the gel was electrophoresed at 40 mA in 1x TBE buffer (10x stock solution 108g Trizma base, 55g Boric acid, 40.0 ml EDTA per litre) for 1.5 hours prior to the loading of sequencing reactions.

2.4 BIOCHEMICAL TECHNIQUES

2.4.1 Tunicamycin treatment of engineered NSO cells to remove N-linked carbohydrate

Prior to full tunicamycin treatment, cells in tissue culture were pre-treated for 6 hours by the addition of 1.0 ml of 1000x stock tunicamycin solution (Boehringer Mannheim 1 mg/ml in 0.1M NaOH) to each litre of cells in culture. For full treatment, the cells were harvested with trypsin and EDTA, centrifuged in 250.0 ml sterile centrifuge pots for 10 minutes at 1500 rpm in a Sorvall RC5B and then resuspended in the same volume of fresh medium containing 1 mg/ml tunicamycin. The cells were replaced into a humid CO_2 incubator at 37°C for 3 days. The resulting aglycosylated antibody was isolated by fast protein liquid chromatography (FPLC, Pharmacia) and the level of aglycosylation assessed by reduced sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

2.4.2 ^{3}H ethanolamine labelling of cells

Transfected or parental CHO cells were harvested from tissue culture utilising trypsin and EDTA, washed in the relevant medium and adjusted to $5x10^4$ cells/ml. Aliquots of 2.0 ml were pipetted into duplicate wells of 6 well tissue culture plates and the plates incubated at 37°C until the cells became 80% confluent. A volume of ^{3}H ethanolamine (1 mCi/ml Amersham) was diluted 1 in 10 in the relevant medium and 0.2 ml was added to each tissue culture well. After an 18 hour incubation period, the cells were harvested, washed twice in PBS and resuspended, in PBS, to a concentration of 10^6 cells/ml. To assess incorporation of ^{3}H ethanolamine into GPI-anchors, the cells were further diluted either 1 in 10 or 1 in 100 with reducing sample buffer for analysis on SDS-polyacrylamide gels alongside unlabelled and ^{14}C labelled rainbow markers (14300-200000 molecular weight, Amersham).

2.4.3 ^{35}S methionine labelling of cells

Cells to be radioactively labelled were harvested from tissue culture flasks with trypsin and EDTA and washed, in methionine-free medium (Gibco/Life Technologies), by centrifugation in a Sorvall RT6000B centrifuge at 1500 rpm for 5 minutes at room temperature. The cells were counted by trypan blue exclusion and corrected to $2x10^5$ cells/ml in methionine-free medium prior to aliquoting a volume of 10.0 ml into a 25 cm^2 tissue culture flask which was laid horizontal to permit adherence. Once the cells had adhered, the medium was removed and 10.0 ml fresh methionine-free medium added and the flask replaced at 37°C in a humid CO_2 incubator for several hours. A 14 µl aliquot of

^{35}S methionine 70% / cysteine 30% (Amersham, ^{35}S methionine 5 mCi per 500 µl / cysteine 2.5 mCi per 500 µl) was added and the incubation continued overnight. After harvesting with trypsin and EDTA and washing the cells in three washes of PBS, the pellets were analysed for ^{35}S incorporation on SDS-polyacrylamide gels alongside unlabelled and ^{14}C labelled rainbow markers.

2.4.4 Phosphatidylinositol specific Phospholipase C (PIPLC) treatment of cells to remove GPI-anchored antigen

Cells to be treated were harvested with trypsin and EDTA prior to three washes in medium containing 1% (v/v) FCS. The cells were resuspended to $2x10^6$ cells/ml in the same medium and for each antigen to be assayed, three aliquots of 150 µl dispensed into sequential wells of a round bottomed 96 well microtitre plate. To one of the wells, 7 µl of PIPLC (Boehringer Mannheim, 308 U/200 µl) was added whilst the remaining two wells were left as controls. The plate was placed at 37°C for 90 minutes and then the cells washed, in situ, with three washes of PBS. To visualise each antigen, all cells were resuspended in 100 µl FACS staining buffer and two wells (one of which was PIPLC treated) were stained with specific FITC-labelled antibodies as described in 2.2.1, whilst the third well was stained with a non-specific antibody or left in PBS. All samples were analysed on a Becton Dickinson FACScan analyser.

2.4.5 Removal of oligosaccharides from antibody by Hydranzinolysis

Utilising a technique derived from an Oxford Glycosystems N-glycan Recovery kit and based on hydrazine cleavage (Patel et al 1993), a volume of antibody equivalent to 5 mg was dialysed twice against one litre changes of water overnight prior to freeze drying in an acid-washed glass ampoule. Hydrazine (0.1 ml) was added and the ampoule heated, in a thermostatically controlled block, to 95°C for 5 hours. The released oligosaccharides were isolated by resuspending the sample in 1.5 ml butanol: ethanol: acetic acid solution (4: 1: 0.5) and passing the mixture through a 1.0 ml Whatman CF-11 cellulose column, previously equilibrated with 4.0 ml of the same solution. After three 1.0 ml washes with butanol: ethanol: acetic acid (4: 1: 0.5), followed by three 1.0 ml washes with butanol: ethanol: water (4: 1: 1) the bound oligosaccharides were finally washed with 1.0 ml of methanol and the column flow stopped. N-reacetylation of the samples occurred by the sequential addition of 1.0 ml methanol and 0.4 ml acetic anhydride, the majority of which was allowed to pass through the column prior to stopping the column flow for 30 minutes. The bound oligosaccharides were again washed four times in 1.0 ml aliquots of butanol: ethanol: water (4: 1: 1) proceeded by a 1.0 ml methanol wash. To elute the free oligosaccharides, the column was washed with two 1.0 ml volumes of 0.2M sodium acetate and the eluate collected. Final treatment of the sample involved the addition of 0.1

ml acetic anhydride and after 30 minutes at room temperature, the addition of 0.1 ml of 0.1M copper acetate in 0.1M acetic acid and a further 30 minute incubation. Passage of the isolated oligosaccharides through a 1.0 ml column of mixed bed ion exchange resins Chelex 100 and AG-50 (pre-washed with water), led to the recovery of water eluted oligosaccharides ready for freeze drying. The oligosaccharides were later analysed by either high-performance anion exchange chromatography (HPAEC, Dionex) or by laser desorption-mass spectrophotometry (LDMS, Finnigan).

2.4.6 Analysis of oligosaccharides by Dionex HPAEC

Separation of oligosaccharides was by Dionex HPAEC using a Carbopak PA-1 column, elution buffer with a linear gradient of 24-36 mM sodium acetate in 125 mM NaOH over 20 minutes and a flow rate of 1.0 ml/minute. Detection of the samples was by pulsed amperometry using a gold electrode. An internal standard of sialyic acid (neuraminic acid) was included to determine relative retention times of the isolated oligosaccharide peaks.

2.4.7 Analysis of oligosaccharides by LDMS

Samples of desialylated oligosaccharides (1-20 pmol) in 1 µl of water were mixed with 1 µl 2,3-dihydroxy benzoic acid (DHB, 10 mg/ml in 50% (w/v) ethanol) as the matrix component and a 1 µl aliquot allowed to dry onto a sample slide. Ionisation by irradiation with a pulse of laser light was followed by analysis with a Lasermat time-of-flight mass spectrophotometer (Finnigan MAT).

2.4.8 Enzymatic removal of carbohydrate from oligosaccharides

These techniques were performed by Robert Lifely and Susan Boyce of the department of Molecular Immunology, Wellcome Research Laboratories, Beckenham and the methods are briefly outlined below. Analysis of oligosaccharides was by Dionex HPAEC and LMDS (Finnigan).

2.4.8.1 Desialylation of oligosaccharides

Oligosaccharides were treated with 40 mM trifluoracetic acid at 80°C for 1 hour under helium. The trifluoracetic acid was removed under a stream of air at 40°C and the sialic acid content measured by Dionex HPAEC.

2.4.8.2 Exoglycosidase treatments of oligosaccharides

Exoglycosidases including bovine testes galactosidases, bovine epididymis fucosidase and jack bean meal N-acetylhexosaminidase were obtained from Oxford Glycosystems. Oligosaccharides (5-10 g) were incubated with enzyme, following the manufacturers

instructions, at 37°C for 48 hours. Samples were purified by passage through a column of mixed bed ion exchange resins, 0.3 ml Dowex AG-50 layered upon 0.3 ml Dowex AG-3 and were eluted with water. The samples were lyophilised prior to analysis on a Dionex HPAEC.

2.4.9 Isolation of NSO-F and CHO-Campath-1H antibody from culture supernatants

Transfected NSO.F and CHO cell culture supernatants were harvested from 8000 litre bioreactors, clarified by centrifugation and passed through 0.2µm filters. The antibodies were captured on protein A-Sepharose columns and eluted with 0.1M citrate buffer (pH 3.0), then bound to S-Sepharose ion-exchange columns and eluted with 20mM citrate-phosphate buffer containing 200mM NaCL. The eluates were concentrated, desalted and monomeric antibody separated from dimeric and polymeric antibody utilising a Superdex 200 size-exclusion column. (Lifely et al 1995). The CHO Campath-1H utilised in assays was of a single clinical trial batch made and lyophilised prior to the start of this project. A single batch of NSO.F Campath-1H was produced from the clone C7F10 9D4-5A11 in October 1993.

2.4.10 Isolation of NSO.S, PF and CF antibody from culture supernatant utilising fast protein liquid chromatography (FPLC)

Transfected NSO cell culture supernatants were collected and pooled after each subculture. The antibody containing fluid was centrifuged in a Sorvall RT6000B centrifuge at 1500 rpm for 5 minutes to remove cell debris and filter sterilised through a 0.2 micron filter prior to storage at 4°C. When 100.0 ml volumes were available, the amount of antibody in the supernatant was assessed by an anti-human Ig ELISA and the antibody purified. A Pharmacia FPLC system was programmed to siphon the supernatant through a 1.0 ml Hi-Trap protein G column at 2.0 ml/minute, wash the column with excess PBS and elute the antibody in 0.1M glycine pH 2.0. Protein G usage minimised bovine Ig contamination from FCS containing supernatants. The resulting eluate was neutralised with 1M Tris-HCl pH 8.8 prior to extensive dialysis against PBS over a 48 hour period. The antibodies were re-sterilised by filtration and stored at 4°C until required. Two batches of NSO.S (#s 1 and 2) were produced in August 1992, two batches of NSO.PF (# 1) in October 1992 and (#2) in February 1993 and one batch of NSO.CF in May (1993). Unless stated, all antibodies utilised in the assays were from the clone C7F10 9D4-5A11.

2.4.11 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Utilising a variation of the method of Laemmli (1970) and a stock solution of pre-mixed 30% (w/v) acrylamide/0.8% (w/v) bis acrylamide, variable percentage acrylamide gels

were prepared for SDS-PAGE analysis. The gel mix was diluted to the desired concentration (usually 10%, therefore 10.0 ml) by the addition of water up to 18.7 ml and the sequential addition of 11.2 ml of 1M Tris-HCl pH 8.8, followed by 0.3 ml 10% (w/v) SDS, 0.1 ml 10% (w/v) ammonium persulphate and 20 µl tetramethylethylenediamine (TEMED, Sigma). After gentle mixing by inversion, the liquid gel was pipetted into a mould of two glass plates, separated by 0.75 mm spacers, sandwiched together in a Hoeffer mini gel apparatus. An overlay of 2.0 ml N-butanol was pipetted onto the gel and the whole left to polymerise. Once solid, the gel surface was rinsed with several washes of water, drained well and a 5% stack gel containing 1.67 ml 30% gel mix, 1.25 ml 1M Tris-HCl pH 6.8, 0.1 ml 10% (w/v) SDS, 50 µl 10% (w/v) ammonium persulphate and 10 µl TEMED in a final volume of 10.0 ml was poured around a 10 slot 0.75 mm toothed comb. To electrophorese samples in a reduced form, 0.65 ml sample buffer (1.88 ml 1M Tris-HCl pH 6.8, 6.0 ml 10% (w/v) SDS, 3.0 ml glycerol and 2.12 ml water) was pre-mixed with 0.1 ml 1.5M dithiothreitol/0.1% (w/v) bromophenol blue and an equal volume added to the test sample (if the samples were to be electrophoresed in a non-reduced form the dithiothreitol was omitted). The sample was heated to 100°C for 5 minutes and cooled on ice prior to electrophoresis at 40 mA in a single strength run buffer of 3.03g Trizma base, 14.42g glycine and 1.0g SDS per litre of water.

2.4.12 Immunoprecipitation of antigens

Pellets of between $5x10^6$ and 10^7 cells were resuspended in 500 µl lysis buffer (0.5g octylglucoside, 2.8 ml 2.5M NaCl, 500 µl 1M Tris-HCl pH 8.0, 200 µl 0.5M EDTA, 250 µl 0.2M phenylmethylsulphonyl fluoride (PMSF), 500 µl aprotinin 2 mg/ml and a small amount of iodoacetamide per 50.0 ml water), mixed well and placed on ice for 30 minutes. Cell debris was removed by centrifugation at 14000 rpm, 4°C for 30 minutes in an Eppendorf refrigerated 5402 centrifuge. Pre-clearing of the lysate was accomplished by a 20 minute end-over-end rotation with 100 µl of 50% (v/v) Sepharose 4B beads which had been previously washed in three changes of 100 µl lysis buffer. Immunoprecipitation of the desired antigen was achieved by exposing the pre-cleared lysate to 20 µl washed 50% (v/v) Sepharose 4B beads which had been pre-coated with a relevant antibody such as Campath 1H $F(ab')_2$, 4 mg/ml. A 30 minute end-over-end rotation at RT was followed by five washes of the Sepharose beads with 5x 1.0 ml aliquots of lysis buffer. To separate precipitated antigens, the antigen linked Sepharose beads were mixed with 20 µl reducing sample buffer and electrophoresed on SDS-polyacrylamide gels alongside either ^{14}C labelled or unlabelled rainbow markers (Amersham). To visualise the antigens, the gel was stained with 1% (w/v) Coomassie blue in 10% (v/v) acetic acid, 45% (v/v) methanol prior to destaining in 7% (v/v) acetic acid, 20% (v/v) methanol. If the cells had been radioactively labelled prior to the immunoprecipitation, the resulting gel was not stained

but soaked in destain solution for 20 minutes, agitated in Amplify solution (Amersham) for 20 minutes, dried onto 3MM filter paper and exposed to photographic film overnight at -70°C.

2.4.13 Western blotting and detection of immunoprecipitated proteins

For antibody detection of specific proteins, SDS-polyacrylamide gels were not stained but the proteins were transferred to nitro-cellulose via the method of western blotting (Towbin et al 1979). The transfer of proteins was accomplished by the use of an Anthos semi-dry blotting apparatus and a blotting buffer composed of 2.9 g glycine, 5.8 g Trizma base, 3.7 ml 10% (w/v) SDS and 20% (v/v) methanol per litre. Prior to the gel being placed in the apparatus, a sandwich of three squares of 3 MM filter paper and one piece of nitro-cellulose paper were cut to the correct size for the gel, soaked in blotting buffer and laid, nitro-cellulose uppermost, onto the cathode face of the apparatus. The gel was removed from the gel template and placed onto the nitro-cellulose paper. A further three pieces of buffered soaked filter paper were overlaid onto the gel and by the use of rolling a glass rod over the stack, air bubbles were carefully removed. The anode face of the blotting apparatus was lowered onto the gel stack and a current of 200 mA applied for 15 minutes. The nitro-cellulose paper bearing the proteins was removed from the apparatus and soaked in 10.0 ml PBS containing 3% (w/v) milk powder (Marvel) overnight at 4°C. The following day, the PBS/milk reagent was replaced with a fresh 10.0 ml aliquot to which was added 30 µl biotinylated antibody of choice, i.e. biotinylated anti-CD4 antibody Q4120 (Sigma). If biotinylated antibody was not available, unlabelled antibody was used at the same dilution. After 1.5 hours of gentle agitation at room temperature, the filter was washed six times in PBS containing 0.1% (v/v) Tween 20 prior to the addition of a 1 in 1000 dilution of the detecting reagent, streptavidin bound horse radish peroxidase (Strep-HRP Sigma). If an unlabelled antibody had been applied previously, the same dilution of an anti-species antibody coupled to HRP was substituted. The visualisation of the specific protein was achieved by utilising an enhanced chemiluminescence, ECL, detection kit (Amersham) as per the manufacturers instructions.

2.5 GRAPHICS

The graphics were devised utilising the Origin (version 3.5) software package. The concentration of antibody giving either 50% of the maximum value specific lysis or 50% inhibition in each experiment is presented at the bottom of each graph or in the associated text.

CHAPTER THREE: RESULTS - MECHANISM OF ACTION OF THE CHO ANTIBODY

3.1 Introduction:

Campath-1H was the trade name of a Glaxo-Wellcome IgG1 'humanised' antibody which recognises the CDw52 antigen on mature lymphocytes, monocytes and macrophages. It is derived from a rat IgG2a, class-switched to IgG2b, monoclonal antibody isolated following the immunisation of rats with human T cells (Introduction, section 1.6). The rat monoclonal antibody, Campath-1G, was found to be particularly effective in vitro at depleting human cells of CDw52 bearing cells prior to transplantation (Hale et al 1987). To overcome the problem of human anti-rat antibody (HARA) responses to Campath-1G in vivo (Dyer et al 1990b), the antibody producing rat B cells were used as the source from which the heavy and light chain genomic DNA sequences, complete with the CDRs of the variable portions, were isolated for 'humanisation' (Reichmann et al 1988). The 'humanised' heavy and light chain DNA's were re-cloned into expression vectors and transfected into rat YO myeloma cells for expression but yields were low. To achieve high expression levels, the cDNA of the heavy and light chains were re-isolated from the myeloma cells and re-cloned into two mammalian expression vectors (Page and Sydenham 1991) and both plasmids were co-transfected into CHO cells for expression. Preliminary ADCC data comparing YO and CHO-derived Campath-1H suggested that the antibodies differed in efficacy in in vitro assays (Crowe et al 1992). A hypothesis was established predicting that the variation might be due to differences in N-linked glycosylation of the Fc portion of the antibody induced by the expressing cell line. To investigate this theory it was thought necessary to define the mode of action of CHO Campath-1H in a limited range of assays.

3.2 Results discussed within this chapter

This chapter describes the characterisation of Campath-1H antibody derived from CHO host cells in order to establish certain criteria on which to compare antibody prepared from different recombinant cell lines (Chapter four). The characterisation has been limited to assays which were considered to be important in the biological function of Campath-1H administration to patients and encompasses ADCC, monocyte mediated inhibition of target cell growth, the engagement of cell surface antigen and carbohydrate analysis. A detailed investigation of each assay attempts to define the cell types involved, the individual IgG FcR used plus concentration and time responses. By the use of CDw52 negative cells, the antigen specificity of these responses was investigated.

Results

3.3 The carbohydrate composition of Campath-IH antibody expressed in CHO cells

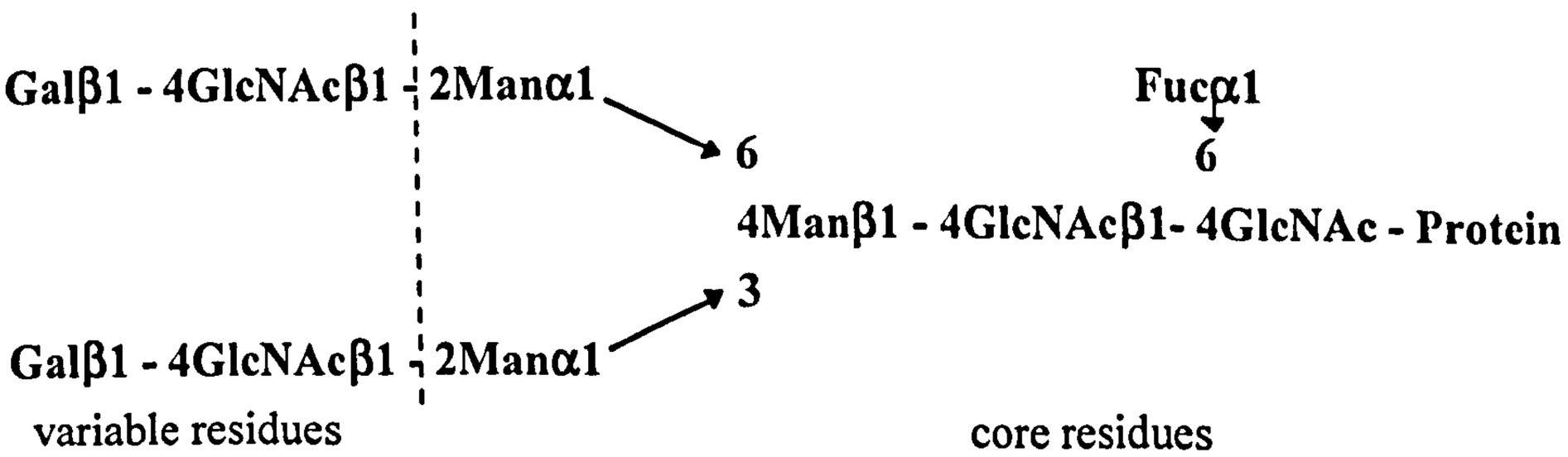
3.3.1 Campath-1H aggregate levels

Samples of Campath-1H clinical trial material which were to be used routinely in the following experiments were sent, for size exclusion HPLC aggregate analysis to determine contaminating levels of polymeric antibody, to Dr. Mark Easton in the Department of Biological Development, Wellcome Research Laboratories, Beckenham, Kent. All samples were found to contain 1.1% polymeric aggregate.

3.3.2 Carbohydrate profile of Campath-1H

Dr. Robert Lifely of the Wellcome Research Laboratories, Beckenham, Kent, determined that within CHO Campath-1H Fc there was a major family of fucosylated N-linked oligosaccharides whose members differed only in terminal galactosylation (Lifely et al 1995). He also isolated a minor non-fucosylated component. The three major peaks obtained from the fucosylated series represented either none, one or two terminal galactose residues. By releasing the monosaccharides from the oligosaccharide complexes, it was shown that Campath-1H contained mannose, galactose, N-acetylglucosamine and fucose in addition to less than 5% N-acetylneuraminic acid. No bisecting GlcNAc residues were identified. The structure determined to be associated with Campath-1H derived from CHO cells is shown in Fig.3.1 and the carbohydrate profile is described in more detail in Chapter four.

Figure. 3.1 A schematic representation of the major carbohydrate structure associated with Campath-1H. The dotted vertical line defines the boundary between variable and core residues.



galactose (Gal),

N-acetylglucosamine (GlcNAc)

mannose (Man)

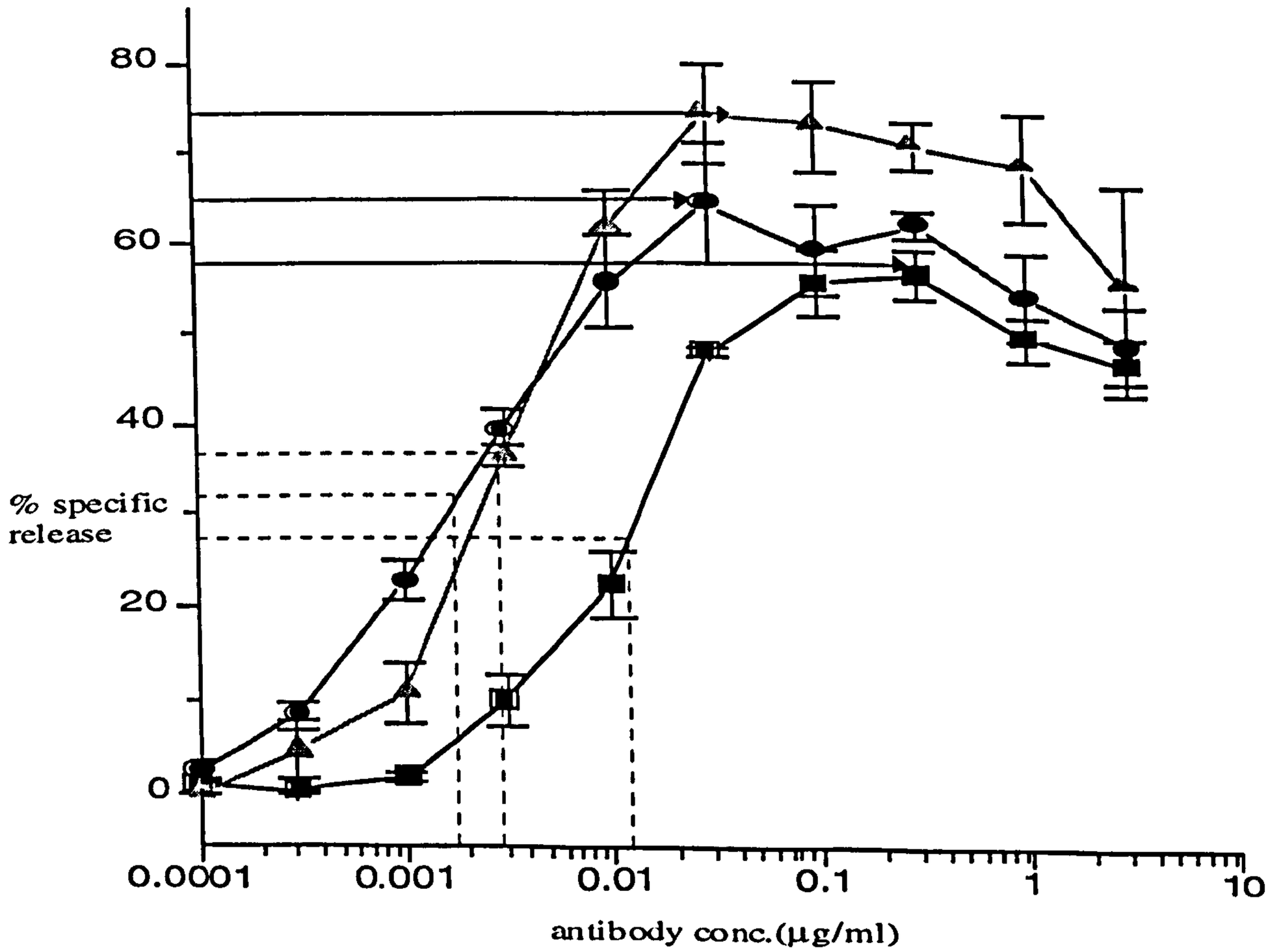
fucose (Fuc)

3.4 The Antibody -Dependent Cell-mediated Cytotoxicity (ADCC) assay

3.4.1 Campath-1H ADCC dose response using Wien 133 cells as targets

ADCC assays in which Campath-1H was diluted over a range of concentrations show that the antibody can direct optimal cell-mediated cytotoxicity of Wien 133 target cells down to a concentration of 0.03 μg/ml (Fig.3.2). The lytic effect decreased as the concentration was reduced below this. Multiple assays indicated that different isolates of effector cells varied in the level of lytic response mediated by Campath-1H and therefore assays were repeated several times with a selection of donor sources. Donors were found to vary in the concentration of Campath-1H required to achieve 50% of maximum specific release with the concentrations ranging between 0.013 and 0.0017 μg/ml.

Figure 3.2 A Campath-1H dose response ADCC assay demonstrating donor variation. Wien 133 cells at $2x10^5$cells/ml were pre-treated with Campath-1H antibody at the amount shown prior to incubation with effector PBMC from three different donors. ■ represents donor J, ● represents donor S and ▲ represents donor I. The data plotted are mean % specific release ± standard errors for each concentration. The solid horizontal lines are maximum specific lysis (75% donor I, 65% donor S and 57% donor J) and the dotted lines are 50% of maximum.



Antibody concentrations leading to 50% of maximum specific lysis are; 0.0028 μg/ml donor I, 0.0017 μg/ml donor S and 0.013 μg/ml donor J.

3.4.2 The importance of effector cell to target cell ratio

Effector to target cell ratios of between 100:1 and 0.5:1 were used to define the appropriate conditions over which to monitor antibody function. The antibody was used at a concentration of 1 μg/ml, a level previously shown to mediate killing of target cells by all donor effector cells. The results (not shown) implied that adequate lysis occurred at effector to target cell ratios of greater than 10:1. Subsequent assays were designed to incorporate a minimum ratio of 25:1, with the targets at 10^4/well and the effectors at $2.5x10^5$/well.

3.4.3 Cell types involved in ADCC

ADCC assays were performed in which cell types within PBMC effector populations were removed and their role in cell-mediated killing investigated. Different ratios of the resulting effector cell populations were used with Campath-1H at 10 μg/ml (Fig.3.3). When CD3 (T) (69.13% of PBLs by FACS) or CD20 (B) (13.94%) positive cells were reduced to 29.27% and 0.87% respectively there was no affect. However, when CD14 positive monocytes were selectively reduced from 17.13% to 3.48% the ADCC response was also reduced. The reduction of CD56 positive cells (from 25.23% to 7.24%) totally ablated the ADCC response suggesting that natural killer (NK) cells are the major cell type involved.

To confirm that the cell type which was critical for the ADCC response resided within the mononuclear cell fraction, further separations of whole blood were performed in which both the mononuclear and polymorphonuclear cell populations were isolated by differential separation on lymphoprep gradients. The two cell populations were utilised as effector cells over a range of effector cell to target cell ratios (Fig.3.4) and the cell morphology was confirmed by Giemsa staining of cytocentrifuge samples (not shown). Within the polymorphonuclear effector population there was an increasing incidence of mononuclear cells at ratios higher than 10:1, and thus any increase in specific lysis could be accounted for by the few mononuclear cells. The most active cell population for killing was confirmed as the mononuclear cell fraction.

Figure 3.3 A Campath-1H ADCC assay demonstrating the effects of PBMC effector cells depleted of various cell types. Wien 133 cells at $2x10^5$cells/ml were treated with 10 µg/ml Campath-1H prior to incubation with various ratios of effector cells depleted with antibody. ■ represents total PBMC, ● represents PBMC minus CD56 positive cells, ▲ represents PBMC minus CD14 positive cells, ▼ represents PBMC minus CD20 positive cells and ◆ represents PBMC minus CD3 positive cells. The data plotted are mean % specific release ± standard errors for each concentration.

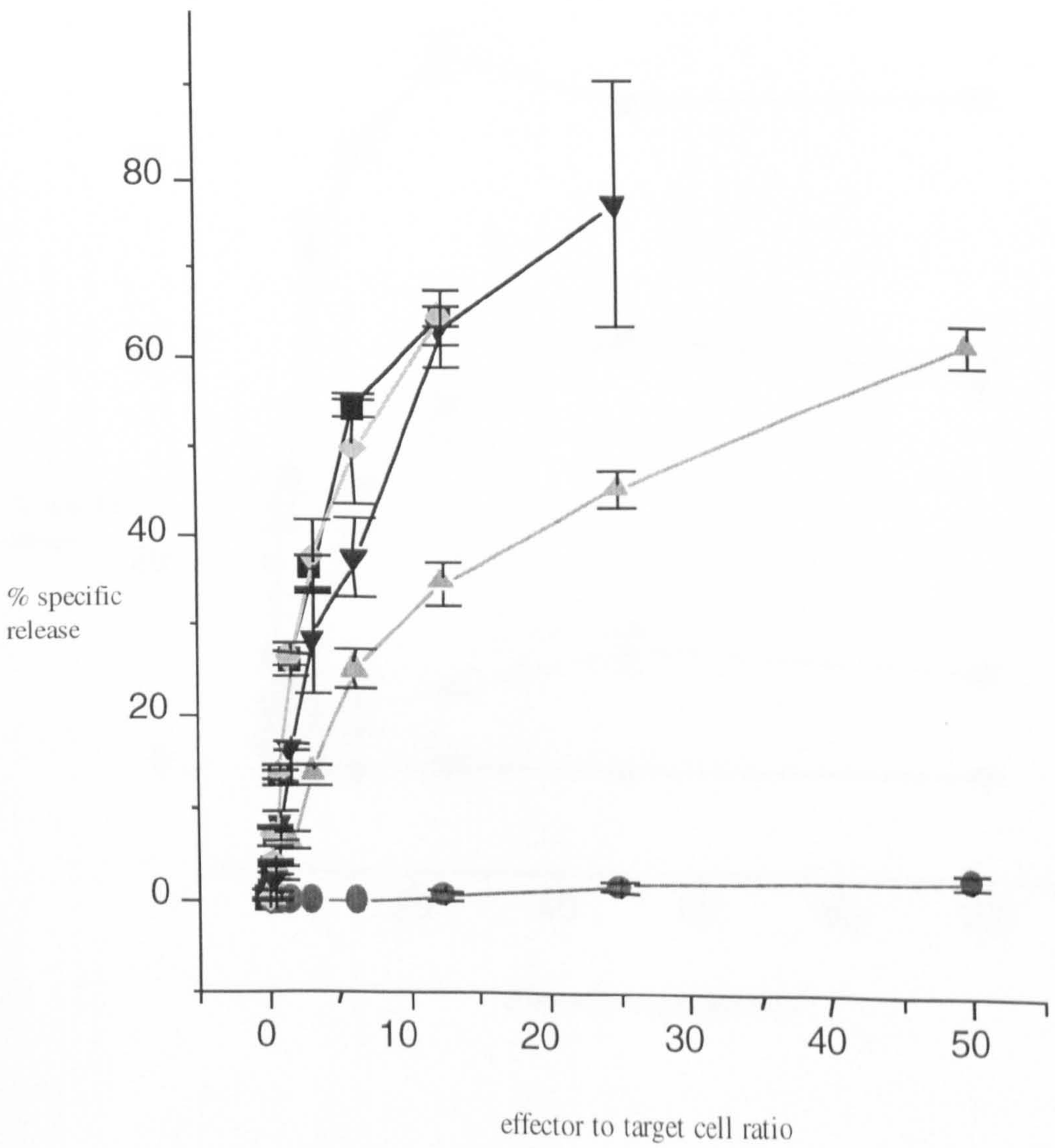
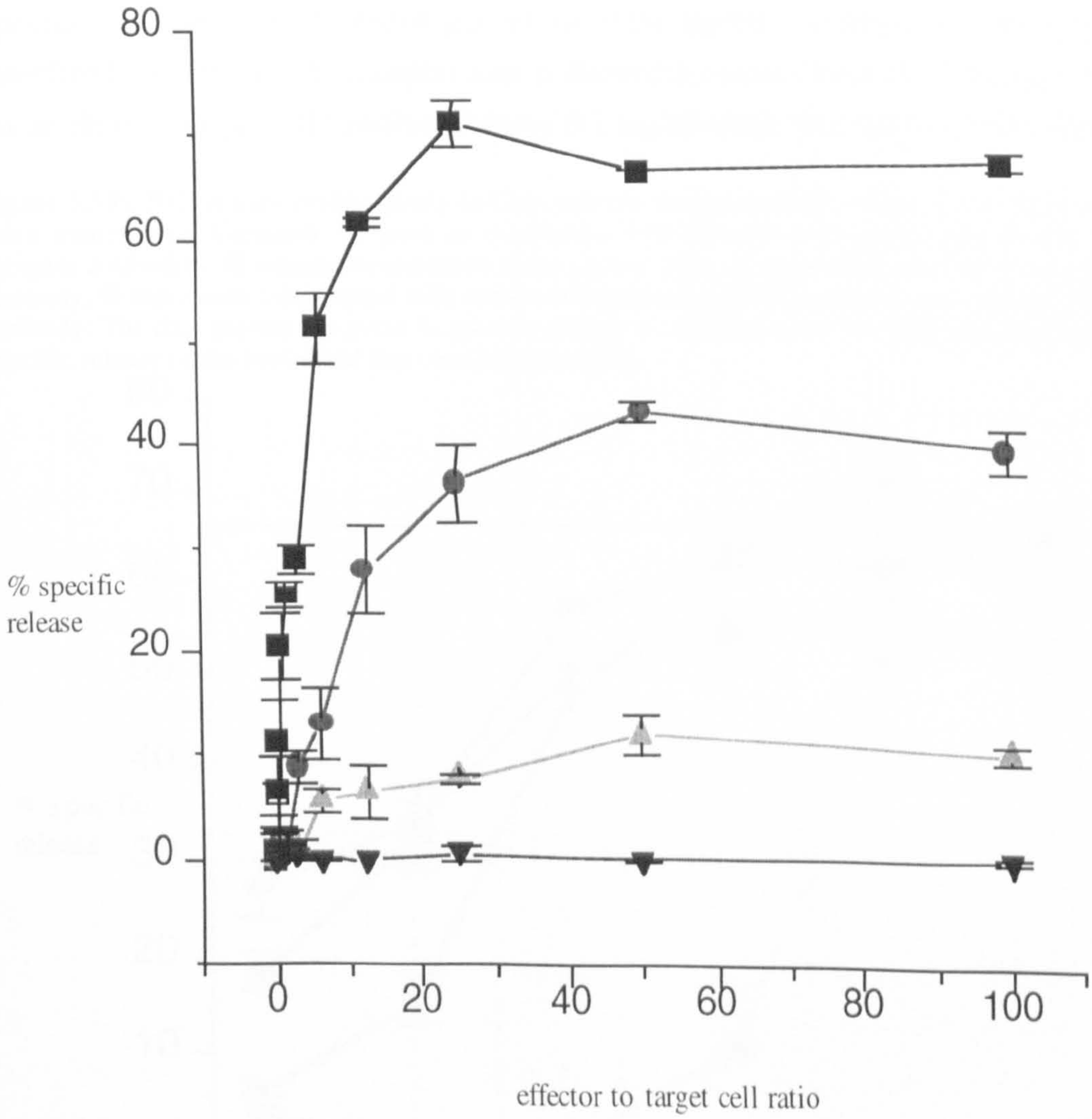


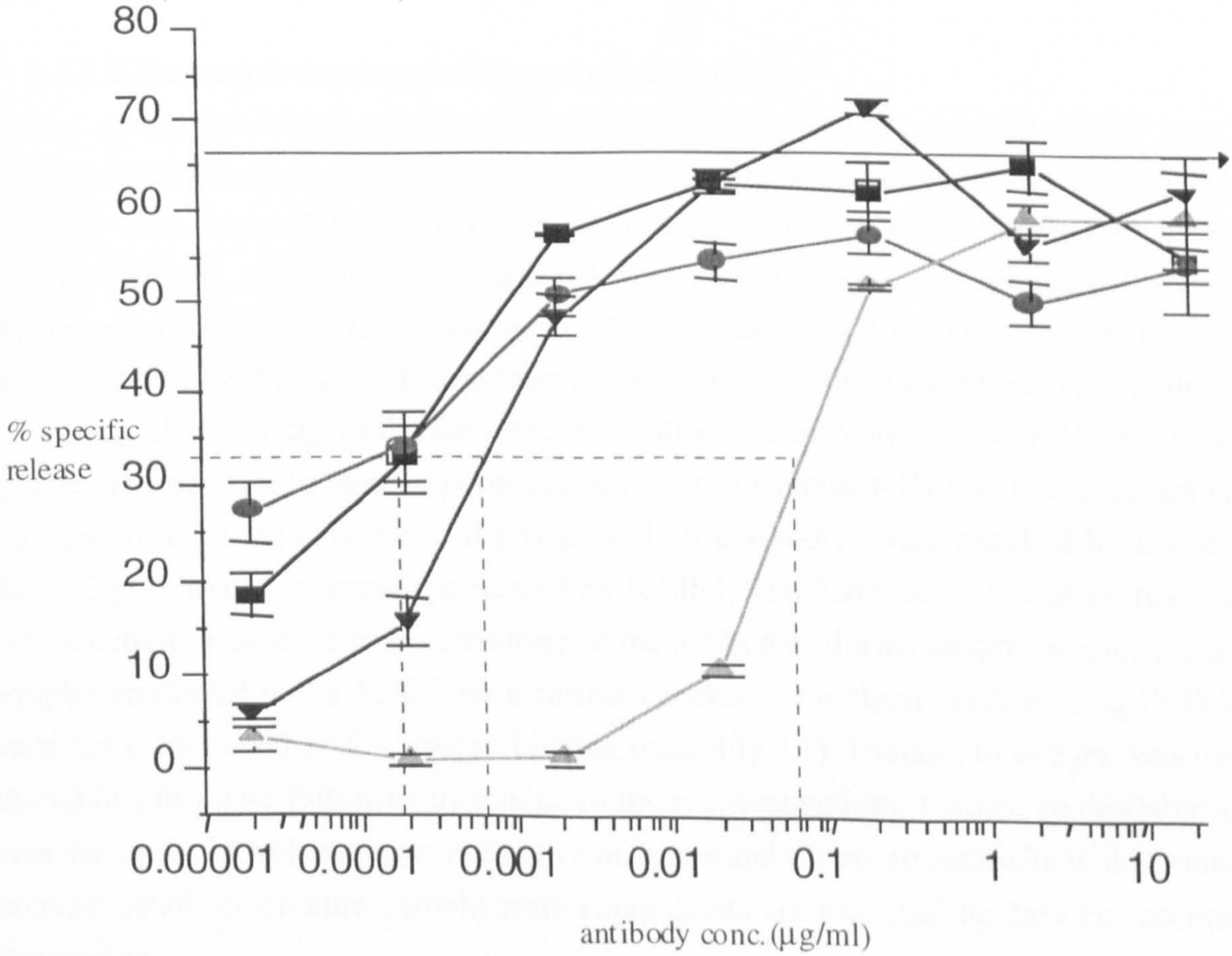
Figure 3.4 A Campath-1H ADCC assay demonstrating the differences between mononuclear and polymorphonuclear cells as effector populations. Wien 133 cells at $2x10^5$cells/ml were treated with Campath-1H or medium prior to incubation with various ratios of either mononuclear or polymorphonuclear effector cells. ■ represents mononuclear cells in the presence of Campath-1H, ● represents polymorphonuclear cells in the presence of Campath-1H, ▲ and ▼ represent mononuclear and polymorphonuclear cells, respectively, in the absence of Campath-1H antibody. The data plotted are mean % specific release ± standard errors for each effector to target cell ratio.



3.4.4 Fc R usage by peripheral blood effector cells

To determine which IgG Fc receptors were most prominently utilised by the PBMC effectors, antibodies (Medarex) specific for IgG Fc receptors were incorporated, (due to cost considerations) at a single dose of 0.5 µg in 10 µl, with effector cells prior to their addition to the ADCC assay. Unfortunately, the anti-Fc reagents were not tested independently prior to the experiments and thus were not demonstrated to influence receptor function. The preliminary data in Fig.3.5 suggests that IgG Fc RIII is the key receptor required for the antibody-mediated killing. Treatment with anti-Fc RIII antibody increased the concentration of Campath-1H required to achieve 50% of maximum specific release from 0.00018 µg/ml to 0.06 µg/ml. Antibodies which specifically interfered with the Fc RI receptor also indicated a minor effect (0.0006 µg/ml required). At levels of Campath-1H antibody above 0.2 µg/ml there was indiscernible inhibition.

Figure 3.5 Fc RIII is used preferentially in Campath-1H-mediated ADCC. Wien 133 cells at $2x10^5$cells/ml were treated with Campath-1H prior to incubation with effector cells previously treated with anti-Fc receptor antibodies. ■ represents untreated mononuclear cells, ▲ represents cells treated with anti-Fc RIII antibody, ● represents cells treated with anti-Fc RII antibody and ▼ represents cells treated with anti-Fc RI antibody. The data plotted are mean % specific release ± standard errors for each concentration. Maximum specific release (solid horizontal line) was taken as 66%.



Antibody concentrations required for 50% of maximum specific release were; 0.00018 µg/ml untreated or when anti-Fc RII was present, 0.0006 µg/ml in the presence of anti-Fc RI and 0.06 µg/ml in the presence of anti-Fc RIII.

3.4.5 Carbohydrate involvement in the ADCC mode of action - Effects of removal

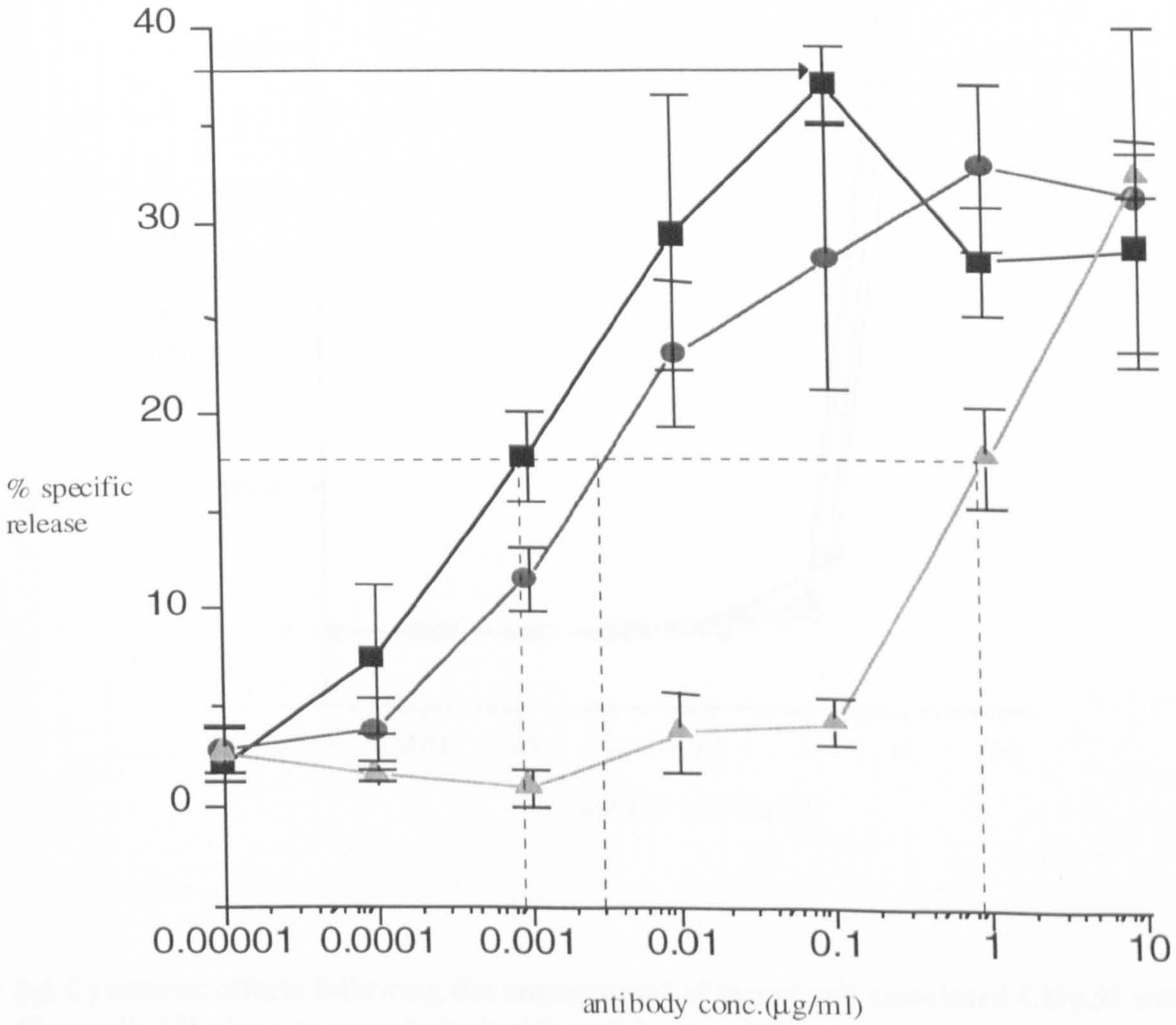
3.4.5.1 Tunicamycin treatment of antibody producing cells

To investigate whether the carbohydrate of Campath-1H Fc CH2 antibody domain was involved in the interaction with the Fc receptor on the ADCC effector cell, batches of CHO cells producing Campath antibody were pre-treated with tunicamycin prior to the antibody being harvested and purified. The resulting purified aglycosylated antibody was compared with the standard Campath-1H, initially by SDS-PAGE to confirm the molecular weight change (data not included) and then in an ADCC assay to determine any loss of activity. Although the actual results are not shown they were similar to those in Fig.3.6, in which purified antibody which had been deglycosylated with N-glycanase was demonstrated to be inactive in ADCC assays when used at 0.1 μg/ml and below. Lysis at higher concentrations of deglycosylated Campath-1H might be due either to carbohydrate remaining after N-glycanase treatment which was of insufficient level to be detected by SDS-PAGE or toxic formulation.

3.4.5.2 Enzymatic treatment of the purified antibody

Further studies investigating the involvement of carbohydrate structures in ADCC were undertaken. The various carbohydrate moieties known to be associated with Campath-1H (Fig.3.1) were sequentially removed, enzymatically, from the whole purified antibody and the resulting antibodies were adjusted to the same protein concentration (280nm absorbance values) prior to utilisation in ADCC assays (Fig.3.6). The data shows that pre-treatment of antibody with hexosaminidase to remove the glucosamine moiety does not greatly alter activity whilst the removal of all of the carbohydrate with N-glycanase ablates the response. In these experiments the control Campath-1H was mock treated at the same time as the experimental groups and all antibodies were checked by Dionex HPAEC prior to use in assays (performed by R.Lifely and S.Boyce). To confirm that the loss of activity was not due to denaturing of the antibodies during enzyme treatment, all samples employed in the ADCC were further checked for antigen binding using ELISA (data not shown) and FACS antigen binding tests (Fig.3.7). Binding to antigen was not altered in either case following treatment. In future investigations it would be desirable to treat the antibody with enzymes to remove mannose and fucose sequentially to determine in more detail which core carbohydrate components are essential for IgG Fc receptor recognition.

3.6 A Campath-1H ADCC dose response assay demonstrating the effects of various carbohydrate modifications. Wien 133 cells at $2x10^5$ cells/ml were pre-treated with dilutions of enzymatically modified Campath-1H prior to mixing with effector cells. ■ represents un-modified Campath-1H, ● represents Campath-1H treated with hexosaminidase to remove N-acetylglucosamine and ▲ represents Campath-1H deglycosylated with N-glycanase. The data plotted are mean % specific release ± standard errors for each concentration. Maximum specific release was taken as 37.5% (solid horizontal line).

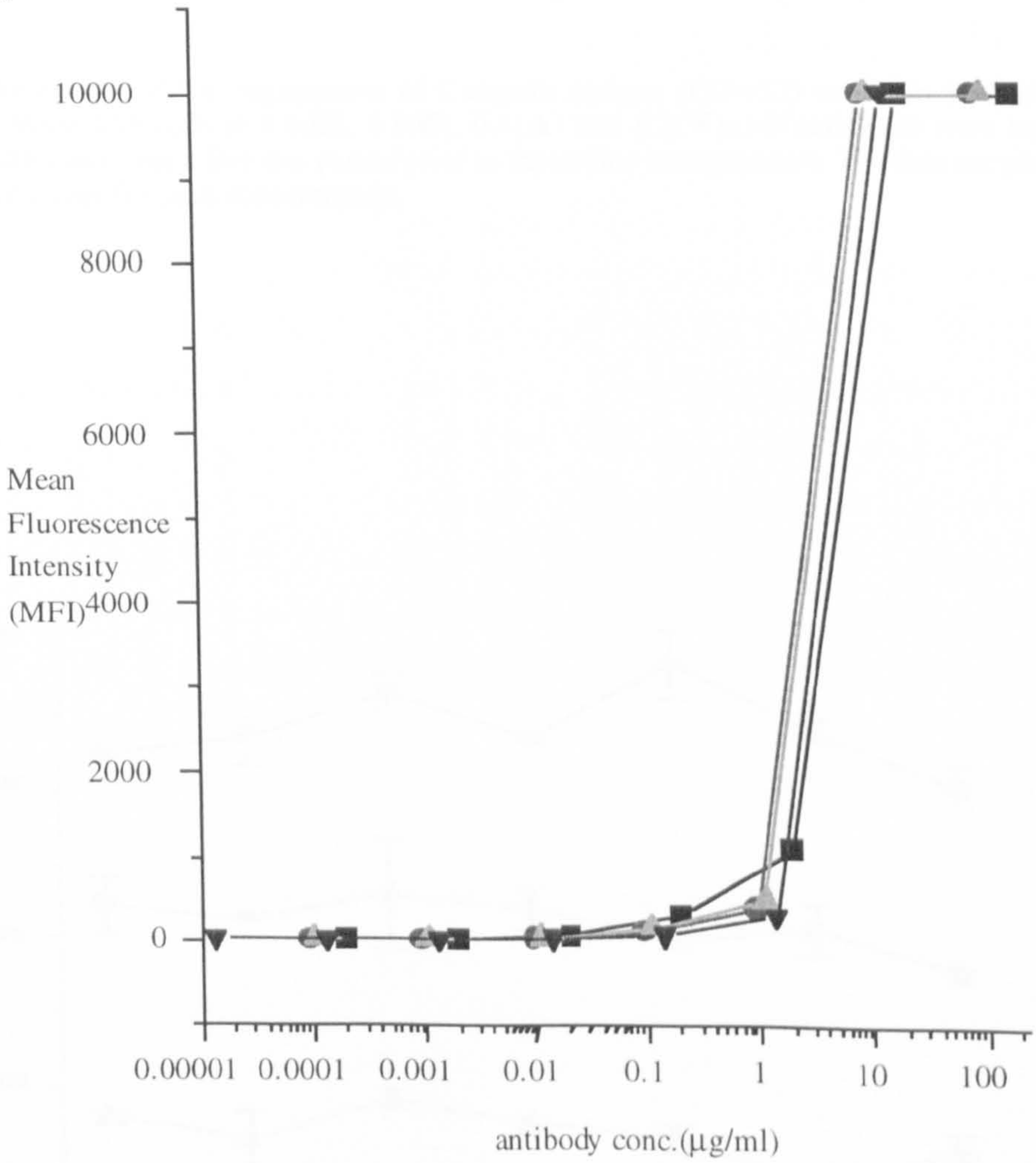


Antibody concentrations required to achieve 50% of maximum specific lysis were; 0.0009 µg/ml for C 1H, 0.003 µg/ml for hexosaminidase treated C 1H and 0.09 µg/ml for deglycosylated C 1H.

For FACS analysis, aliquots of antibody were taken and diluted in a series of ten fold dilutions (from their original concentration). The dilutions were mixed with antigen expressing cells and binding detected as described in the Materials and Methods section.

When mean fluorescence intensity values were plotted against antibody concentration the binding curves obtained (Fig.3.7) were very similar.

Figure 3.7 The mean fluorescence intensity (MFI) of Campath-1H dilutions after enzymatic treatment of the antibody. Dilutions (50µl) of control ■, mock hexosaminidase treated ●, hexosaminidase treated ▲ or deglycosylated CHO Campath-1H antibody ▼ were mixed with $5x10^4$ Wien 133 cells prior to detection with anti-human IgG-FITC conjugate. Results are expressed as the MFI at different antibody concentrations.



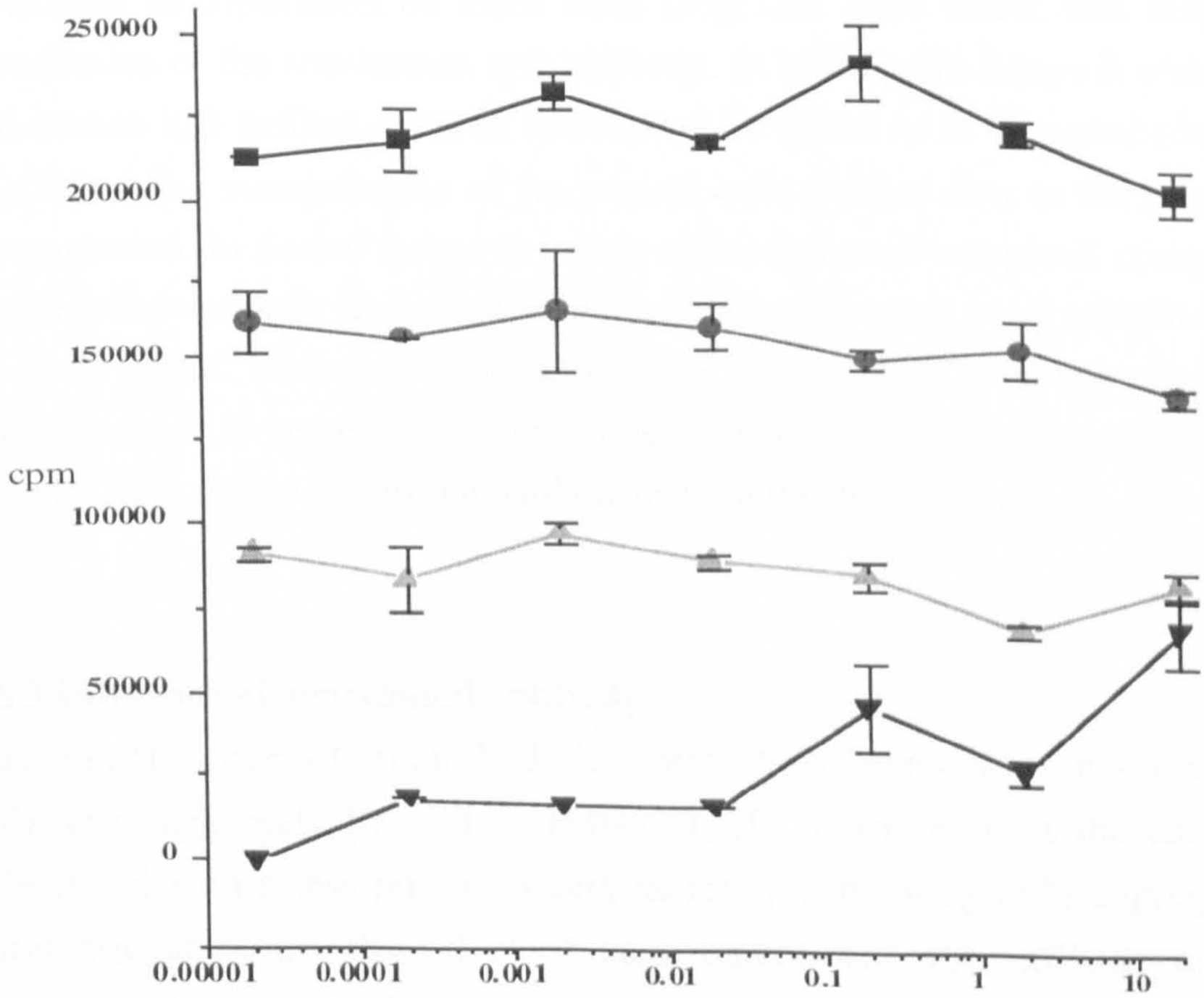
3.5 Cytostatic effects following the engagement of target cell associated CDw52 with Campath-1H alone or crosslinked with anti-human IgG

3.5.1 Optimisation of target cell numbers in the assay

Four concentrations of Wien 133 cells were exposed to Campath-1H antibody dilutions for five days, prior to monitoring thymidine incorporation (Fig.3.8). At the cell densities of 1.6, 0.8 and $0.4x10^4$ cells/ml, thymidine incorporation reduced as the cell number decreased. This was independent of the presence or absence of Campath-1H antibody. As expected, the highest density cells incorporated thymidine at the highest level. Cells at

the lowest density ($0.2x10^4$/ml) grew erratically and labelled poorly even in the absence of antibody. Similar assays in which CDw52 transfected Jurkat cells (see Chapter five for details of the clone 3B/11) were utilised as targets gave similar results (data not shown). Subsequent experiments utilising either target cell line were performed with the cells at densities of either $4x10^3$ cells/well in 50 µl (Wien 133 cells) or $1x10^4$ cells/well in 50 µl (transfected and parental Jurkat cells) with Campath dilutions at 50 µl well.

Figure 3.8 The effects of the engagement of Campath antigen (CDw52) on Wien 133 target cells by Campath-1H. Wien 133 cells at 1.6(■), 0.8(●), 0.4(▲) and 0.2(▼)$x10^4$ cells/well were incubated with Campath-1H dilutions over a five day period prior to thymidine incorporation. The data are plotted as mean cpm ± standard errors for each concentration.



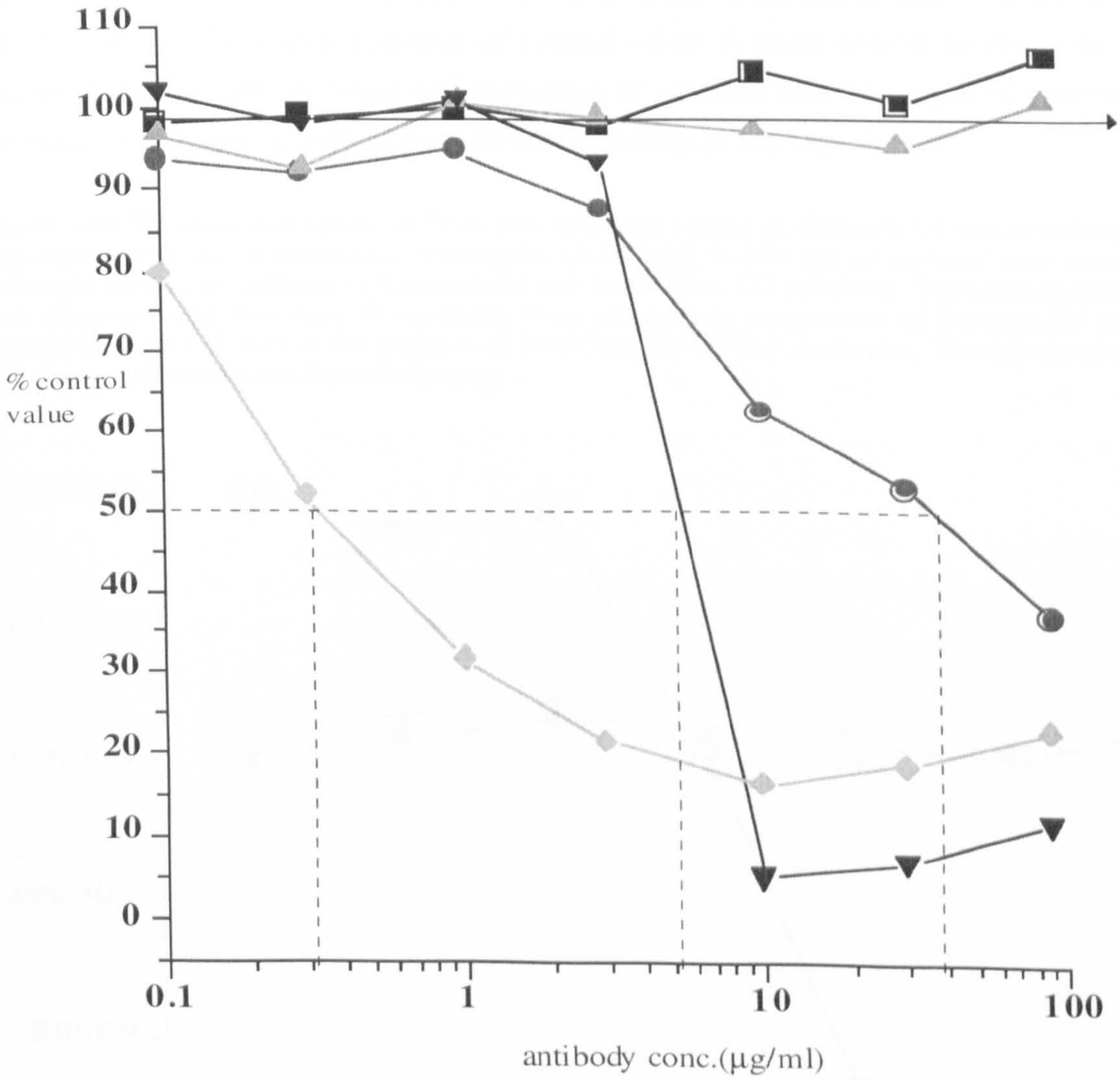
3.5.2 Effects of Campath-1H concentration in the assay

Wendy Rowan (Department of Cell Biology) had shown that over a five day period, utilising Wien 133 cells as targets and Campath-1H at a concentration of 20 μg/ml in the presence of anti-human IgG (20 μg/ml), the cells stopped growing and apoptosed (Rowan et al 1998). Unfortunately, Wien 133 CDw52 negative variants were not available as controls at this time, so utilising her results as a basis, experiments were devised to examine the effect of Campath-1H concentration on transfected cells. Jurkat J6 cells, which had been transfected with the cloned cDNA for CDw52 and had been shown by FACS to have high Campath antigen expression (Chapter five), were used as positive controls in experiments which determined the effects of engaging and crosslinking the cell surface antigen. The parental untransfected cell line served as the negative control. The plates were incubated at 37°C for five days before thymidine incorporation was measured and the results were calculated as a percentage of the control value to aid comparisons between the two cell lines. When Campath antigen was expressed at high levels on the cell surface, as in the case of Jurkat cells expressing CDw52 (3B/11), Campath-1H antibody had a concentration dependent inhibitory effect on the final thymidine incorporation of those cells (Fig.3.9). This effect was amplified by the introduction of the anti-human IgG antibody. In subsequent assays it was found that the anti-human IgG antibody was as effective at 20 μg/ml as at 40 μg/ml (data not shown). The thymidine incorporation of the control cells did not alter in the presence of either concentration. In further assays in which antibodies were compared at single doses, both the targeting antibody and crosslinking antibody, if present, were utilised at 50 μl/well of 40 or 20 μg/ml. When both antigen positive and negative cells were included, the data was expressed as percentage control values to standardise the comparisons between the two cell lines. There are no standard errors with this form of data.

3.5.3 Influence of aggregated antibody

Campath-IH clinical trial antibody had previously been demonstrated to contain 1.1% polymeric aggregate by HPLC. However, if the antibody in the above assays was substituted with a less pure Campath antibody containing 80% aggregate i.e. di- and polymeric antibody, the effect of engagement with the antibody alone was more exaggerated (Fig.3.9). This suggests that the mode of action was dependent upon crosslinking the antigen as no effector cells were involved.

Figure 3.9 The effects of engagement of the Campath antigen (CDw52), on parental or transfected Jurkat cells, with Campath-1H in the absence or presence of anti-human IgG. Parental Jurkat cells or Jurkat cells transfected with CDw52 cDNA (10^4 cells/well) were incubated with 50 µl Campath-1H dilutions, as well as with or without anti-human IgG at 40 µg/ml, 50 µl, for five days prior to thymidine incorporation. ■ represents parental Jurkat cells in the presence of Campath-1H dilution's, ● represents transfected Jurkat 3/B11 cells with Campath-1H dilution's, ▲ represents parental Jurkat cells in the presence of both Campath-1H and anti-human IgG, ▼ represents transfected Jurkat 3/B11 cells in the presence of both Campath-1H and anti-human IgG and ◆ represents transfected Jurkat cells in the presence of aggregated Campath-1H only. The data are plotted as mean % of the control value and are typical of several assays. Maximum control value was 100% (solid horizontal line).



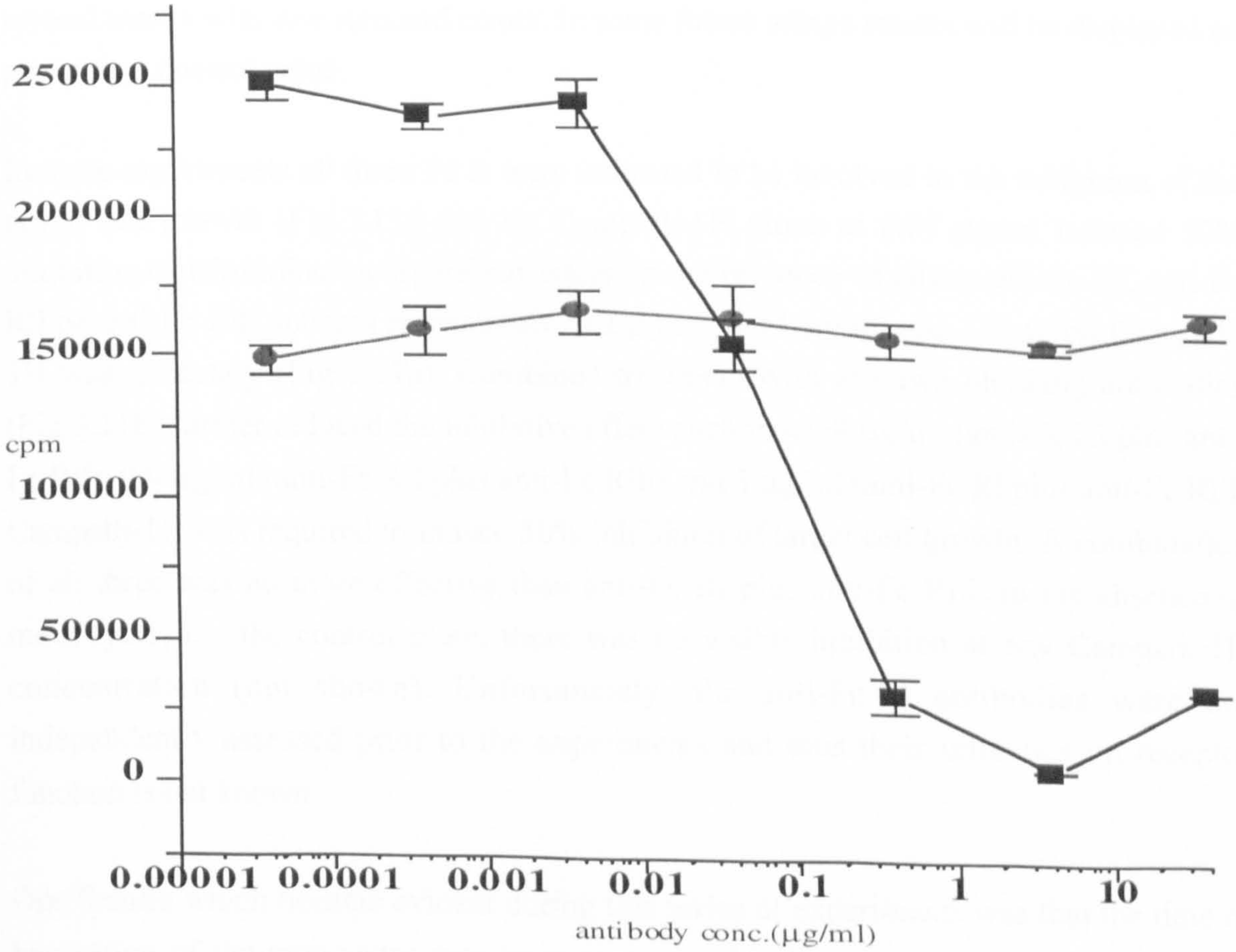
Antibody concentrations giving 50% inhibition were; 0.32 µg/ml for aggregated C 1H alone with 3/B11 cells, 5.2 µg/ml for C 1H plus anti-HuIg with 3/B11 cells and 39 µg/ml for C 1H alone with 3/B11 cells.

3.6 Campath-1H monocyte-mediated growth inhibition of target cells assay

3.6.1 Campath-1H concentration response

To establish a concentration of Campath-1H at which monocytes reproducibly blocked the growth of a Campath antigen expressing target cell line, a range of antibody dilutions were investigated (Fig.3.10). Inhibition of target cell thymidine uptake was optimal at Campath-1H concentrations greater than 0.4 μg/ml (50% inhibition at 0.1 μg/ml) and negligible in the absence of monocytes. At antibody concentrations less than 0.04 μg/ml the target cell thymidine incorporation was enhanced by the presence of monocytes. This effect may be a consequence of monocyte cytokine production and is masked when results are calculated as percentage of control value. A more critical factor in the assay proved to be the ratio of target cell to monocyte numbers and the length of time that the monocytes had been in culture prior to the beginning of the experiment.

Figure 3.10 The thymidine uptake of Wien 133 cells over a range of Campath-1H concentrations in the presence or absence of monocytes. Monocytes ($2x10^4$/well in 100 μl) or medium were precultured overnight prior to the addition of Campath-1H and $4x10^3$ Wien 133 cells/well. Thymidine incorporation was monitored after five days. ● represents Wien 133 cells in the presence of Campath-1H whilst ■ represents Wien 133 cells in the presence of both Campath-1H and monocytes. The data are plotted as mean cpm ± standard errors for each dilution.



3.6.2 Optimisation of monocyte to target cell ratio

Assays were designed to aid the determination of the optimal monocyte to target cell ratio to use routinely in the assays. As monocyte cell numbers were limited (due to the lack of donors prepared to give large amounts of blood) the criteria were established by the collation of several assays (data not shown). It was found that a ratio of at least 2.5 monocytes to each target cell was required, with higher ratios giving more consistent results. Thus, for all subsequent assays, the monocytes were utilised at a minimum of $1.0x10^4$/well and the targets at $4.0x10^3$ cells/well.

3.6.3 Monocyte Fc receptor usage

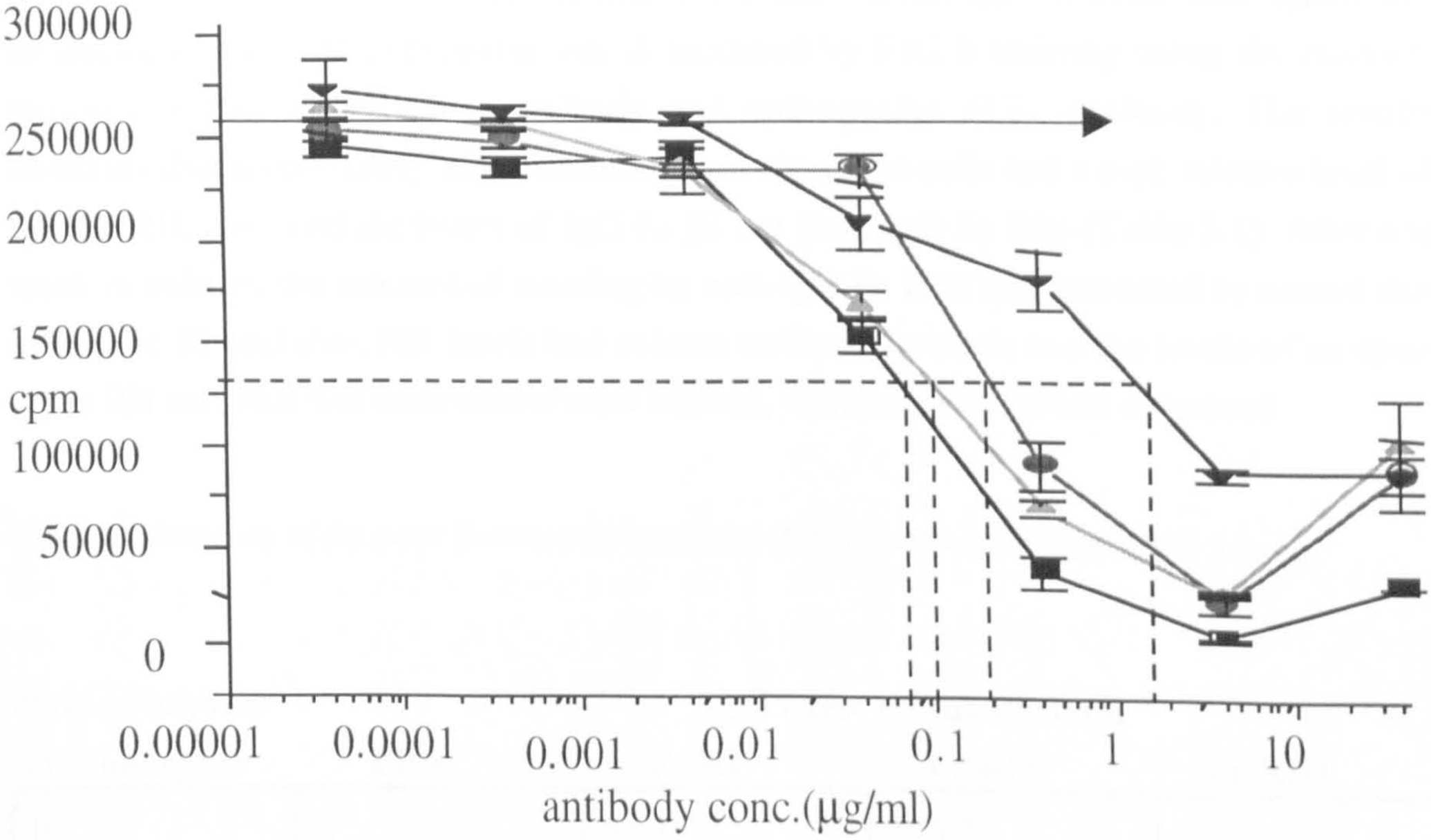
Monocytes display an array of IgG Fc receptors on their surface (van de Winkel and Capel 1993) all, or some, of which could be involved in the growth inhibition effect of monocytes on the antibody coated target cells. To investigate individual receptor involvement, anti-Fc R antibodies obtained from Medarex were incorporated as blocking reagents within the assay, as in the ADCC experiments. The influence of single, combinations or no blocking antibody were explored. The preliminary results are displayed as counts per minute of ^3H thymidine incorporation and are representative of several assays with low standard errors. In some future assays results will be displayed as percentage control value.

In these experiments all three Fc R were indicated to be involved in the inhibition of the target cell growth (Fig.3.11a and b). Campath-1H alone at 0.07 µg/ml induced 50% inhibition of thymidine incorporation whilst in the presence of either anti-Fc RI, anti-Fc RII or anti-Fc RIII antisera respectively, 0.2 µg/ml, 0.11 µg/ml and 1.5 µg/ml Campath-1H was necessary (Fig 3.11a). Combined treatment with any two blocking antibodies (Fig 3.11b) further reduced the inhibitive effect such that 0.4 µg/ml (anti-Fc RI plus anti-Fc RII), 0.9 µg/ml (anti-Fc RII plus anti-Fc RIII) and 3 µg/ml (anti-Fc RI plus anti-Fc RIII) Campath-1H was required to induce 50% inhibition of target cell growth. A combination of all three was no more effective than anti-Fc RI plus anti-Fc RIII. In the absence of monocytes, i.e. the control plate, there was no visible inhibition at any Campath-1H concentration (not shown). Unfortunately, the anti-Fc R antibodies were not independently assessed prior to the experiments and thus their influence on receptor function is not known.

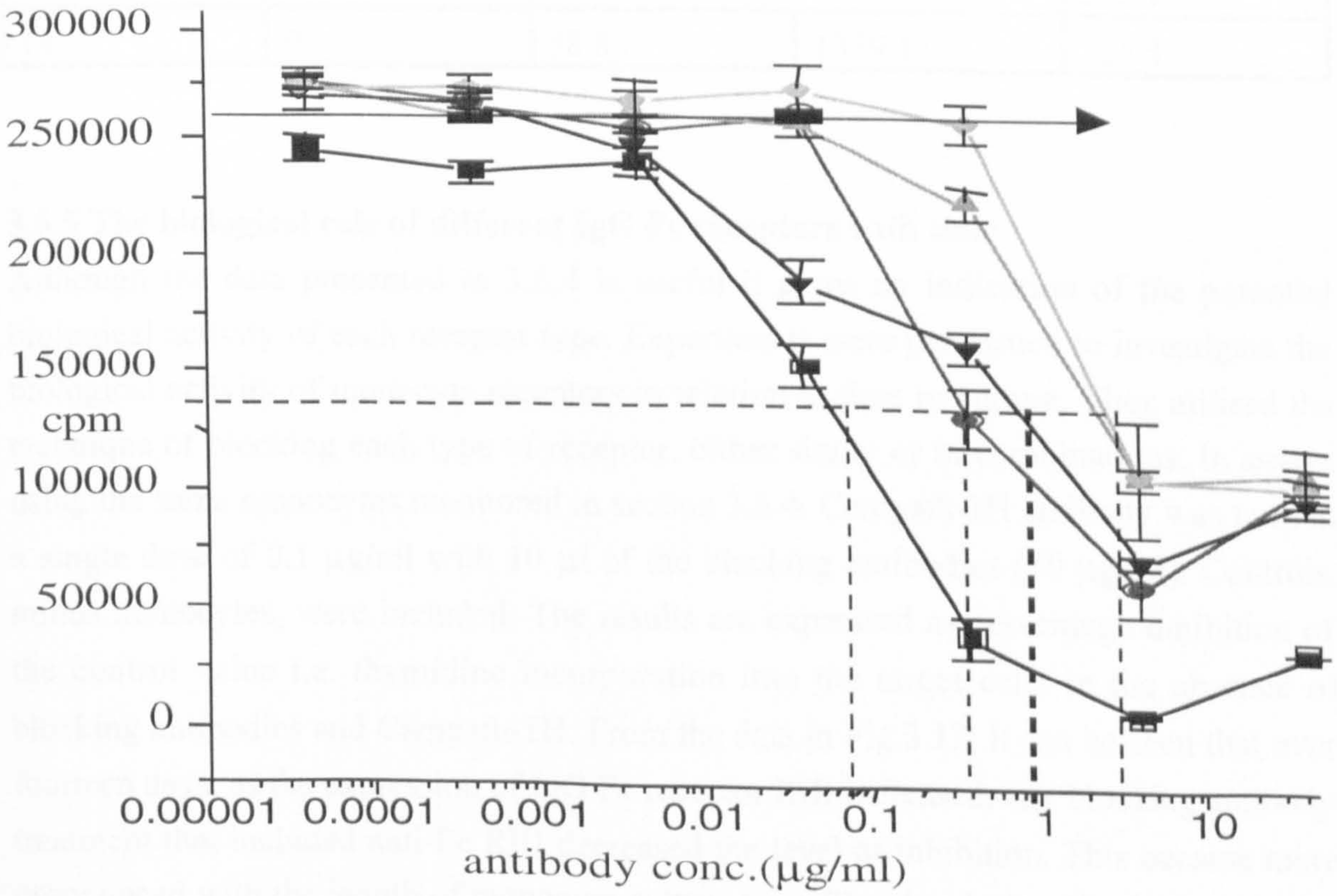
One feature which became evident during this series of experiments was that the time of harvesting of the monocytes was important, possibly because the display of receptors varied with time in culture.

Figure 3.11 A monocyte mediated Wien 133 cell inhibition assay over a range of Campath-1H dilutions, in which anti-IgG Fc receptor antibodies are used to block the monocyte Fc receptors. Monocytes ($1.5x10^4$/well) were preincubated overnight prior to the addition of $4x10^3$ Wien 133 cells and 50 µl Campath-1H. Various anti-Fc antibodies (10 µl, 50 µg/ml) were included during the five days prior to measuring thymidine incorporation. The following symbols represent; a) ■ untreated control, ● anti-Fc RI, ▲ anti-Fc RII, ▼ anti-Fc RIII. b) ■ untreated control, ● anti-Fc RI and RII, ▲ anti-Fc RI and RIII, ▼ anti-Fc RII and RIII , ◆ anti-Fc R1, RII and RIII. The data are plotted as mean cpm ± standard errors for each dilution. Maximum control value was taken as 260,000 cpm (solid horizontal line).

a).



b).



3.6.4 The display of monocyte receptors with time in culture

To investigate the range of IgG Fc receptors displayed on the cell surface at any one time, large amounts of human blood were collected from donors and monocytes set up in culture for various time periods. On days 0, 7 and 14 of culture the monocytes were harvested, counted and aliquoted into 96 well flat bottomed plates for monocyte inhibition assays (as described in Materials and Methods). At each time point the monocytes IgG Fc R expression was determined by FACS staining using the relevant mouse anti-IgG Fc receptor antibody and anti-species FITC-antibody. The results indicated that immediately after isolation from blood the cells had a high relative level of IgG Fc RII, intermediate levels of IgG Fc RI but little IgG Fc RIII (Table 3.1). After one week in culture, the amount of staining by anti-IgG Fc RIII had increased to almost that of IgG Fc RI and also, RII levels had at least trebled. By week two the levels of receptor types RII and RIII had increased almost tenfold, whilst that of RI had decreased.

Table 3.1. Summary of the mean fluorescence intensities (MFI) of each IgG receptor with time

Date of harvest of monocytes	MFI (test minus control) PBS	Fc RI	Fc RII	Fc RIII
0	0	65.3	143.77	18.1
7	0	71.01	494.0	58.31
14	0	38.83	1339.15	125.8

3.6.5 The biological role of different IgG Fc receptors with time

Although the data presented in 3.6.4 is useful it gives no indication of the potential biological activity of each receptor type. Experiments were performed to investigate the biological activity of monocyte receptors in relation to time in culture. They utilised the technique of blocking each type of receptor, either singly or in combinations, in assays using the same monocytes monitored in section 3.6.4. Campath-1H antibody was used at a single dose of 0.1 μg/ml with 10 μl of the blocking antibodies (50 μg/ml). Controls, minus monocytes, were included. The results are expressed as percentage inhibition of the control value i.e. thymidine incorporation into the target cells in the absence of blocking antibodies and Campath-1H. From the data in Fig.3.12, it can be seen that over fourteen days, as the expression of IgG Fc receptor RIII increased, any blocking antibody treatment that included anti-Fc RIII decreased the level of inhibition. This became more pronounced with the length of monocyte culture time. The day 0 plot (Fig.3.12a) implies that the inhibition effect seen in the medium control sample may be largely due to

mechanisms independent of IgG Fc receptors, but is still dependent on the presence of antibody and monocytes. Fc RIII may only be playing a minor role on fresh monocytes, since anti-Fc RIII antibody alone has the same effect as combinations with either anti-Fc RI, anti-Fc RII or in combinations of all three. By day 7 (Fig.3.12b) the IgG receptor RIII alone had increased in influence on the inhibition of the target cells. The combination treatments of anti-Fc RI or RII antibodies, with anti-Fc RIII, were more than additive at this point especially in the case of Fc RI. At the day 14 time point (Fig.3.12c) the influence of RIII was even greater, but RI and RII were also contributing in that the control inhibition effect was totally ablated by a combination of anti-Fc RI, RII and RIII or partially ablated by a combination of anti-Fc RI and RIII. As a consequence of this data, routine assays utilised monocytes which had been in culture for between one to two weeks prior to the experiment and the phenotypes of the receptors present were monitored by FACS analysis on the day of monocyte harvest.

3.6.6 Effects of γ-IFN on the monocyte assay

The above data suggests that IgG Fc RI has an increasingly synergistic effect with RIII when the monocytes have been cultured for longer than one week. Since it has been postulated that γ-IFN increases the activity of IgG Fc RI (van Schie et al 1992), γ-IFN treatment of the older monocytes may enhance this effect. To test the hypothesis, 10 µl of either medium or γ-IFN at $3x10^5$-$0.05x10^5$ units/ml was included throughout the assay period (Fig.3.13). A single sub-optimal dose of 0.1 µg/ml Campath-1H was utilised (0.07 µg/ml gave 50% inhibition in Fig 3.11). The results show that, in the presence of monocytes, the level of Campath-1H employed in the assay did not result in inhibition of target cell thymidine uptake as expected (see Fig 3.11) however, there was a dramatic increase in inhibition on the addition of γ-IFN to Campath-1H. In the absence of monocytes, γ-IFN alone or in combination with Campath-1H both enhanced the target cell thymidine uptake over the majority of concentrations tested. Unfortunately, the monocytes were not assessed for Fc RI levels immediately before and after treatment and therefore it has not been confirmed that the receptor expression was altered in any way or indeed shown that Fc RI was the causative agent.

Figure 3.12 The effects of monocytes which had been in culture for various lengths of time on standard Campath-1H induced, monocyte mediated growth inhibition of Wien 133 cells assays. Medium or monocytes ($1.5x10^4$/well fresh, one week or two weeks in culture) were preincubated overnight prior to the addition of $4x10^3$ Wien 133 cells, 50 µl Campath-1H (0.01 µg/ml) and if present, 10 µl anti-Fc antibody (50 µg/ml). The symbols represent ● monocytes plus Wien 133 cells and Campath-1H, ◐ monocytes plus Wien 133 cells only, ◑ medium plus Wien 133 cells and ○ medium plus Wien 133 cells and Campath-1H,. The data are plotted as mean % of the control value and are typical of several assays. The receptors antibodies are abbreviated to aFc 1 for anti-Fc RI, aFc 2 for anti-Fc RII and aFc 3 for anti-Fc RIII.

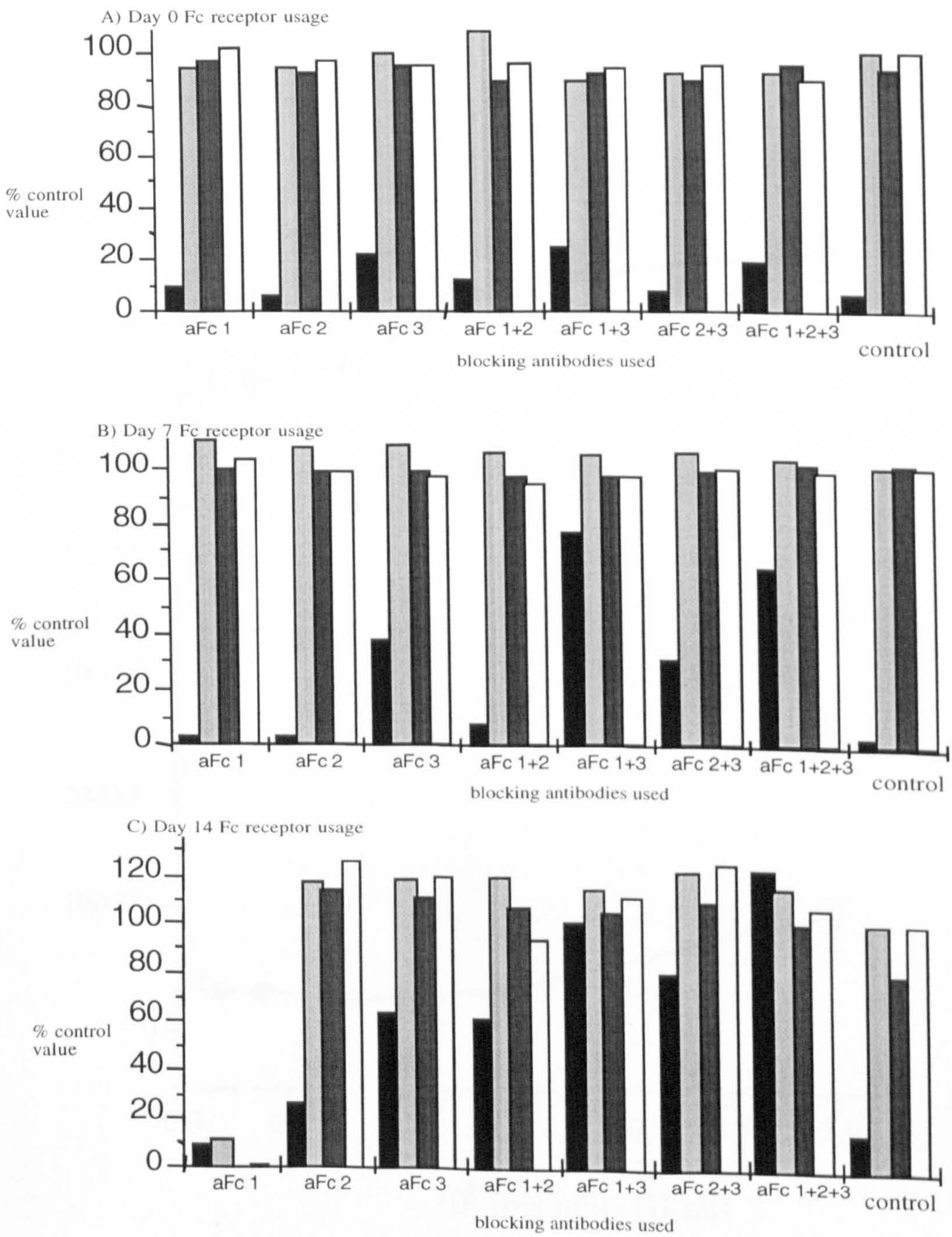
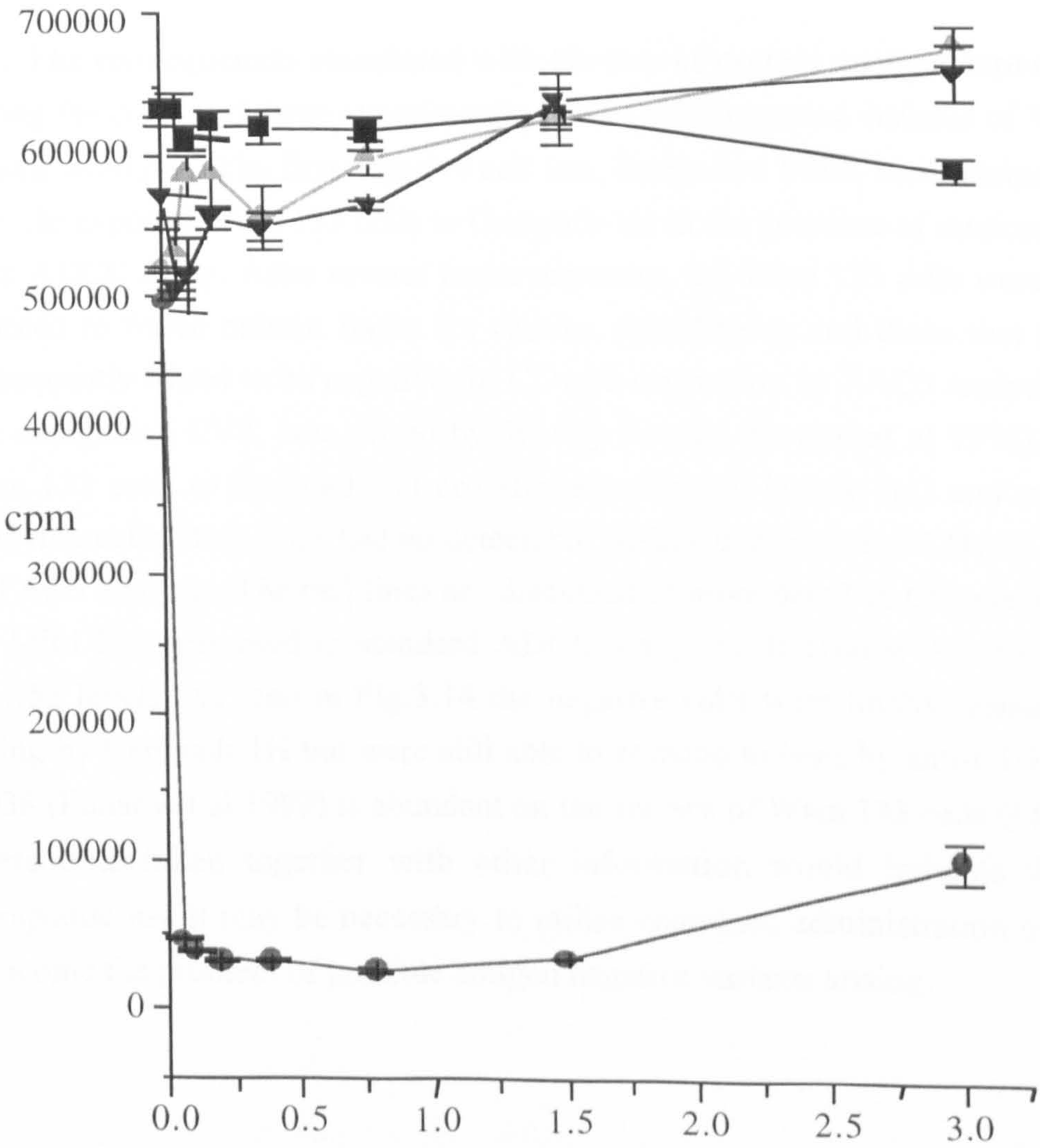


Figure 3.13 The effects of dilutions of γ-IFN on a cytostasis assay. Monocytes ($1.5x10^4$/well) were preincubated overnight prior to the addition of $4x10^3$ Wien 133 cells, 50 µl Campath-1H at 0.1 µg/ml and dilution's of γ-IFN (10 µl). Thymidine incorporation was monitored at day five. ■ represents monocytes plus Wien 133 cells and IFN, ● represents monocytes plus Wien 133 cells, Campath-1H and IFN, ▲ represents medium plus Wien 133 cells and IFN whilst ▼ represents medium plus Wien 133 cells, Campath-1H and IFN. The data plotted are mean cpm ± standard errors for each IFN dilution.



3.6.7 Effects of GM-CSF on the monocyte assay

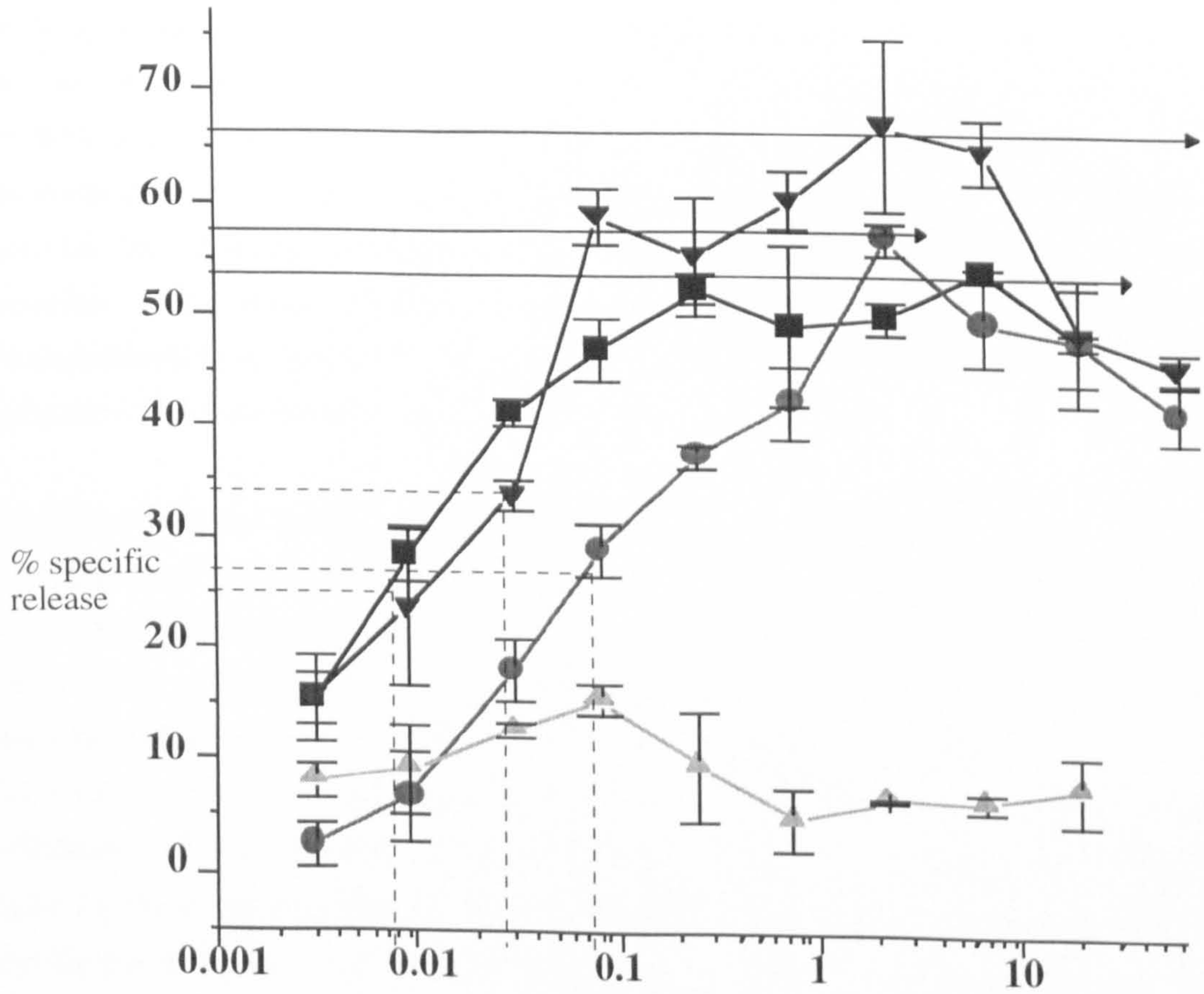
GM-CSF is documented to increase the activity of Fc RII receptors on neutrophils and eosinophils (Bianchi et al 1989, Fanger et al 1989) and it was postulated that it may have a similar affect on monocytes. However, when GM-CSF (10 µl of $2x10^3$-$0.31x10^3$ units/ml) was incorporated into monocyte assays no change in efficiency of target cell inhibition was detected (data not shown). The experiments were repeated several times but changes in the rate or level of inhibition were never detected and therefore the experiments were discontinued.

3.7 The generation of CDw52 negative cells

3.7.1 The consequences associated with the loss of surface antigen expression

During the course of these experiments, two CDw52 negative variants of Wien 133 cells became available. The first negative cell line, designated MR4, was obtained by Dr. John Tite. He exposed Wien 133 cells to Campath-1H in the presence of mononuclear cells as in an ADCC assay. After several hours exposure, the Wien 133 cells were retrieved and returned to tissue culture flasks for routine subculturing and those that survived were subsequently found to be negative for CDw52 expression by FACS analysis. The second line designated CV4, was raised by Wendy Rowan (Rowan et al 1998). She exposed Wien 133 cells to Campath-1H crosslinked with anti-human IgG and subcultured the surviving cells. Both lines had no detectable surface expression of CDw52 when checked by FACS analysis. The cell lines are discussed in more detail in Chapter five. However, the MR4 line was used in standard ADCC assays to determine the effects of reduced CDw52 levels. As seen in Fig.3.14 the negative cells were totally resistant to directed killing by Campath-1H but were still able to respond to lysis by anti-CD38. The antigen CD38 (Funaro et al 1993) is abundant on the surface of Wien 133 cells (Ellis et al 1995). These data taken together with other information would indicate that for future therapeutic use it may be necessary to utilise combined administration of antibodies to overcome the problem of possible antigen negative variants arising.

Figure 3.14. The affect of antigen expression on a Campath-1H mediated ADCC assay. CDw52 positive and negative (MR4) cells were mixed with either Campath-1H or anti-CD38 antibody dilutions for 1 hour prior to the addition of PBMC effector cells at a 25:1 ratio. ^{51}Cr was measured in the supernatants five hours later. ■ Wien 133 cells plus Campath-1H, ● Wien 133 cells plus anti-CD38 antibody, ▲ MR4 cells plus Campath-1H and ▼ MR4 cells plus anti-CD38 antibody. The results are expressed as percentage specific release values ± standard errors. Maximum release values (solid horizontal lines) are taken as 66% for MR4 cells plus anti-CD38, 57% for Wien 133 cells plus anti-CD38 and 53% for Wien 133 cells plus Campath-1H. MR4 cells in the presence of Campath-1H only led to a maximum specific release of 15%.



Concentrations of antibody required to generate 50% of maximum release were 0.0075 µg/ml for Wien cells plus Campath-1H, 0.028 µg/ml for MR4 cells plus anti-CD38 and 0.075 µg/ml for Wien 133 cells plus anti-CD38.

3.8 Discussion

The aim of the work described in this chapter was to investigate and establish criteria for future analysis comparing the Glaxo-Wellcome product Campath-1H expressed in and purified from CHO cells with ensuing forms of Campath-1H antibodies produced in alternative expression systems. Experiments, performed by Dr. Robert Lifely, clarified the carbohydrate composition of the CHO Campath-1H Fc fragment and revealed that the majority of isolated oligosaccharide structures were members of a single fucosylated family which varied only in terminal galactosylation, a minor family of non-fucosylated oligosaccharides was also present (Lifely et al 1995). The removal of the carbohydrate by either tunicamycin treatment of the expressing cells in culture or the enzymatic removal by N-glycanase treatment of the purified antibody led to the loss of activity in several, but not all, of the designated assays and confirmed the previous data of Leatherbarrow et al (1985) and Rademacher et al (1988). Partial and sequential removal of selected monosaccharides i.e. glucosamine, was demonstrated to have an minor effect on ADCC activity but this aspect of the project was not pursued and should be returned to if possible. The data obtained from the hexosaminidase treatment is however complementary to that of Boyd (et al 1995) who showed that removal of Campath-1H galactose residues did not alter ADCC activity.

The first assay to be fully investigated using CHO Campath-1H was the ADCC assay in which the cell type primarily responsible for the lysis of antigen bearing cells was found to be the CD56 positive NK cell residing within the mononuclear cell fraction. As the target cells were labelled prior to their addition to the assay, lysis was interpreted as specific and so care was needed with choice of control. All assays incorporated controls for non-specific lysis (no antibody), total lysis (detergent) and medium controls (no effectors). The mechanism of resulting target cell lysis was not defined but was thought to be by the route of perforin-mediated damage to the target cell membrane (Salcedo et al 1993). For efficient lysis to occur, the ratio of effector to target cell was demonstrated to be optimal at at least 10:1 and the antibody was required to have the majority of the carbohydrate moiety intact. To be competent to induce target cell lysis, single doses of the antibody were used within the range 10-0.1 µg/ml depending on the type of response required. The use of anti-Fc R antibodies suggested that the effector cell utilised Fc RIII to couple itself, via the antibody, to the target cell displaying the Campath antigen on its surface. The Fc RI receptor was utilised to a minor level possibly through monocyte interactions. This specific data however, must be taken with caution as the blocking antibodies were not shown, independently, to influence receptor function. Mononuclear effector cells isolated from different donors displayed a range of responses to the single antibody and consequently it was necessary to repeat each assay with a range of donors.

The reason for the differences between donors was not determined but may be due to receptor polymorphism (van de Winkel and Capel 1993, Sanders et al 1995, Koene et al 1997). However, all donors did give ADCC responses over the same Campath-1H concentration range. For ease of comparison, results were found to be optimally expressed in terms of percentage specific release. This enabled data to be compared as dose of Campath required to achieve 50% of maximum target cell lysis.

In control experiments, minus effector cells, it became clear that at low cell densities it was possible to observe cytostasis of the target cells in the presence of Campath-1H antibody. Such effects could be monitored by tritiated thymidine incorporation in a 5 day assay. Once again care was required with choice of control as in this assay cells surviving at day five were radiolabelled and reduced counts were interpreted as cytostasis. It was necessary to incorporate controls with either no targeting or crosslinking antibody as well as cells with no antigen. Cytostasis was enhanced by deliberate cross-linking of the humanised antibody with anti-human IgG. Furthermore, highly aggregated samples of Campath-1H were particularly effective at mediating this effect. Experiments outlined in both Chapter three and four suggest that these effects are independent of glycosylation as deglycosylated Campath was demonstrated to be as active in these assays as the intact antibody and may represent the effects of merely cross-linking the CDw52 antigen on the surface of the target cell. It has been shown that cross-linking CDw52 in other systems leads to the generation of intra-cellular signals (Rowan et al 1995), a feature in common with several GPI-anchored cell surface molecules. Studies have show that GPI-anchored membrane molecules are enriched in glycolipid enriched membrane domains with members of the Src-Kinase family (Shenoy-Scaria et al 1993 and 1994), potentially explaining the ability of a GPI-anchored protein to mediate signalling events.

Monocytes reside within the mononuclear cell population and they were demonstrated, by depletion, to have minor effects in the short five hour ADCC assays. However, as a cell type, they were demonstrated to be more important in longer assays in which target cell thymidine incorporation was monitored after a fixed time. As with previous assays controls had to be carefully selected. In response to treatment of CDw52 bearing target Wien 133 cells with Campath-1H at doses greater than 0.4 μg/ml, monocytes were able to inhibit the thymidine incorporation of those cells over a five day period. A lower dose of 0.1 μg/ml was found to be more suitable for sub-optimal responses in situations where increases of activity were sought. Such a situation was in the use of γ-IFN (van Schie et al 1992) to augment the contribution of Fc RI to the inhibitory effects of a sub-optimal level of Campath-1H. Attempts to demonstrate similar effects on Fc RII with GM-CSF (Fanger et al 1989, Bianci et al 1989) were not successful. To obtain consistently

reproducible results it was necessary to utilise the monocytes and target cells at a ratio of 2.5:1, with the monocytes having been in culture for no more than two to three weeks. It was indicated in preliminary experiments, using the same anti-Fc R antibodies as those in the ADCC experiments, that the individual IgG receptors present on the monocytes changed during time in culture and for this reason monocytes were strictly monitored, by FACS analysis for receptor type, prior to each assay. Freshly isolated monocytes were indicated to utilise mainly Fc RI and RIII receptors to mediate antibody redirected cytostasis with minor use of Fc RII. After one or two weeks in culture, monocyte Fc RI and RII contributed synergistically with Fc RIII to produce the inhibition effect demonstrated. As with the ADCC data these results should be accepted with caution.

Finally, the antigen specificity of these effects was investigated by utilising antigen negative variants of the Wien 133 cell line derived by experimental conditions selecting for the loss of expression of the CDw52 antigen on their surface (Rowan et al 1998). Clones of the negative variant MR4 were placed into ADCC assays alongside their positive parental line to monitor differences entirely due to level of antigen expression. The negative MR4 cells were found to be resistant to killing by CHO Campath-1H over the range 20-0.002 μg/ml but still susceptible to killing by anti-CD38. The derivation of antigen negative variants was an unexpected consequence of Campath-1H treatment and highlighted the possibility of this occurring within patient groups. Some data from ongoing clinical trials (Brett et al 1996a, Hertenstein et al 1995) does indicate that this does occur in a small percentage of people. Perhaps for therapeutic use, antibodies should be utilised as cocktails to avoid this event arising. Recent data (Rawstron et al 1999) also indicates that some patients may possess low numbers of antigen negative cells prior to treatment with Campath which are selected for during therapy.

Utilising the data from these experiments, in vitro assays were fine tuned for the future comparison of Campath-1H antibodies isolated from various expression systems. The assays studied were those involving mechanisms deemed to be important in the in vivo therapeutic use of Campath-1H in which antibody was required to purge the patient, selectively, of CDw52 bearing cells. Therefore, assays in which cell death or growth inhibition of CDw52 bearing cells occurred via the engagement of human effector mechanisms or by antibody recognition of the antigen, were chosen for investigation. It may be that not all of these mechanisms are harnessed in in vivo therapy with Campath-1H but they do provide a variety of assays over which to compare the efficacy of the individual antibodies in in vitro models.

CHAPTER FOUR: RESULTS - CLONING AND EXPRESSION OF CAMPATH-1H ANTIBODY IN NSO CELLS: COMPARISON WITH CHO-DERIVED ANTIBODY

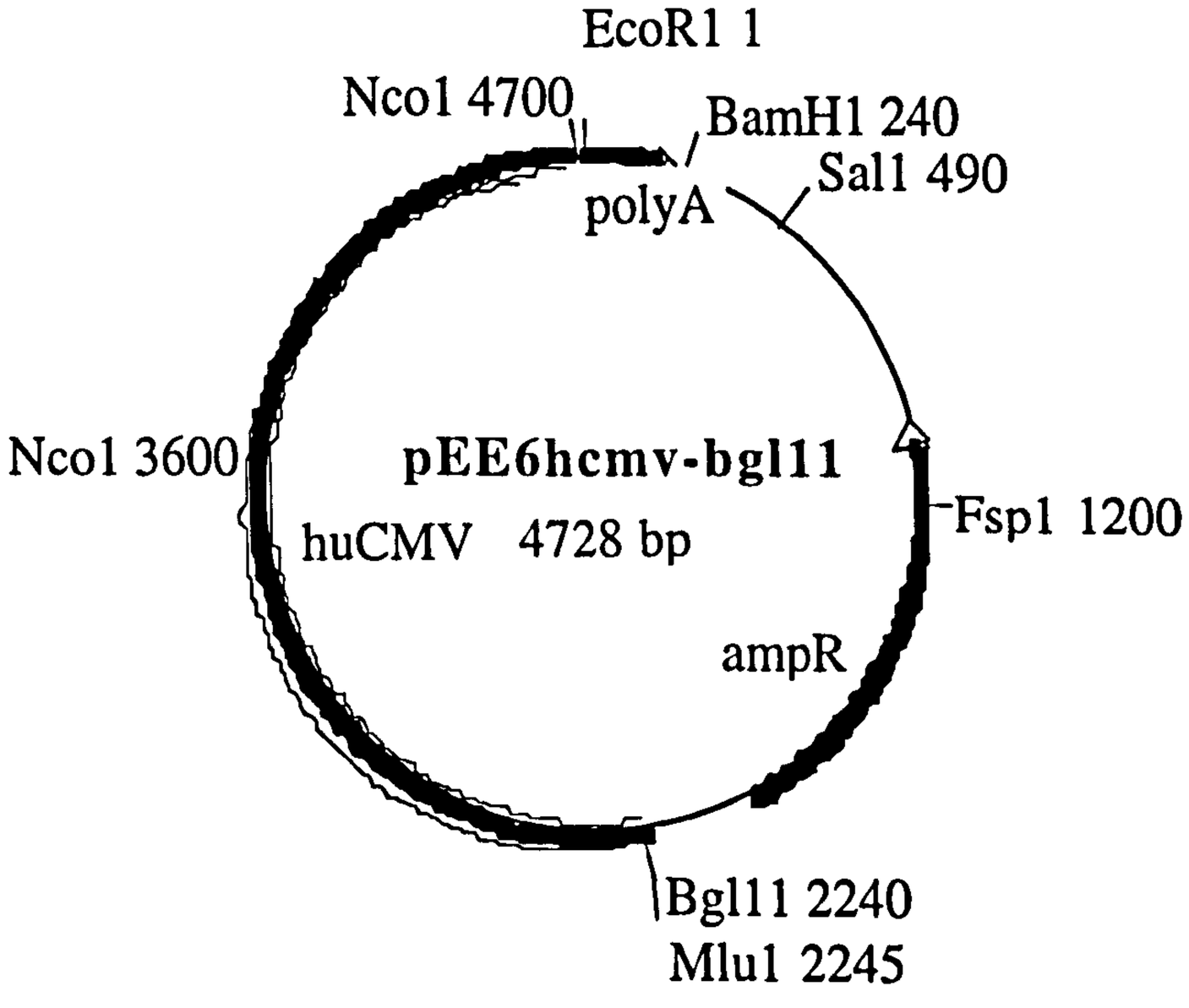
4.1 Introduction

Studies using different Campath-1H antibody preparations had revealed a reproducible difference in the level of lytic response in ADCC assays between antibody sourced from CHO and YO host cell lines (Crowe et al 1992). Additional experiments demonstrated that the enzymatic or tunicamycin-induced removal of N-linked carbohydrate from CHO-derived Campath-1H antibody was sufficient to substantially reduce, or totally ablate, functional activity in several biological assays (Chapter three). Thus it was hypothesised that different host cell lines were capable of adding different post-translational modifications, such as alternative oligosaccharide moieties, to the antibody. The changes in glycosylation were postulated to affect antibody interaction with receptors on effector cells and thus explain the variation in donor lytic response.

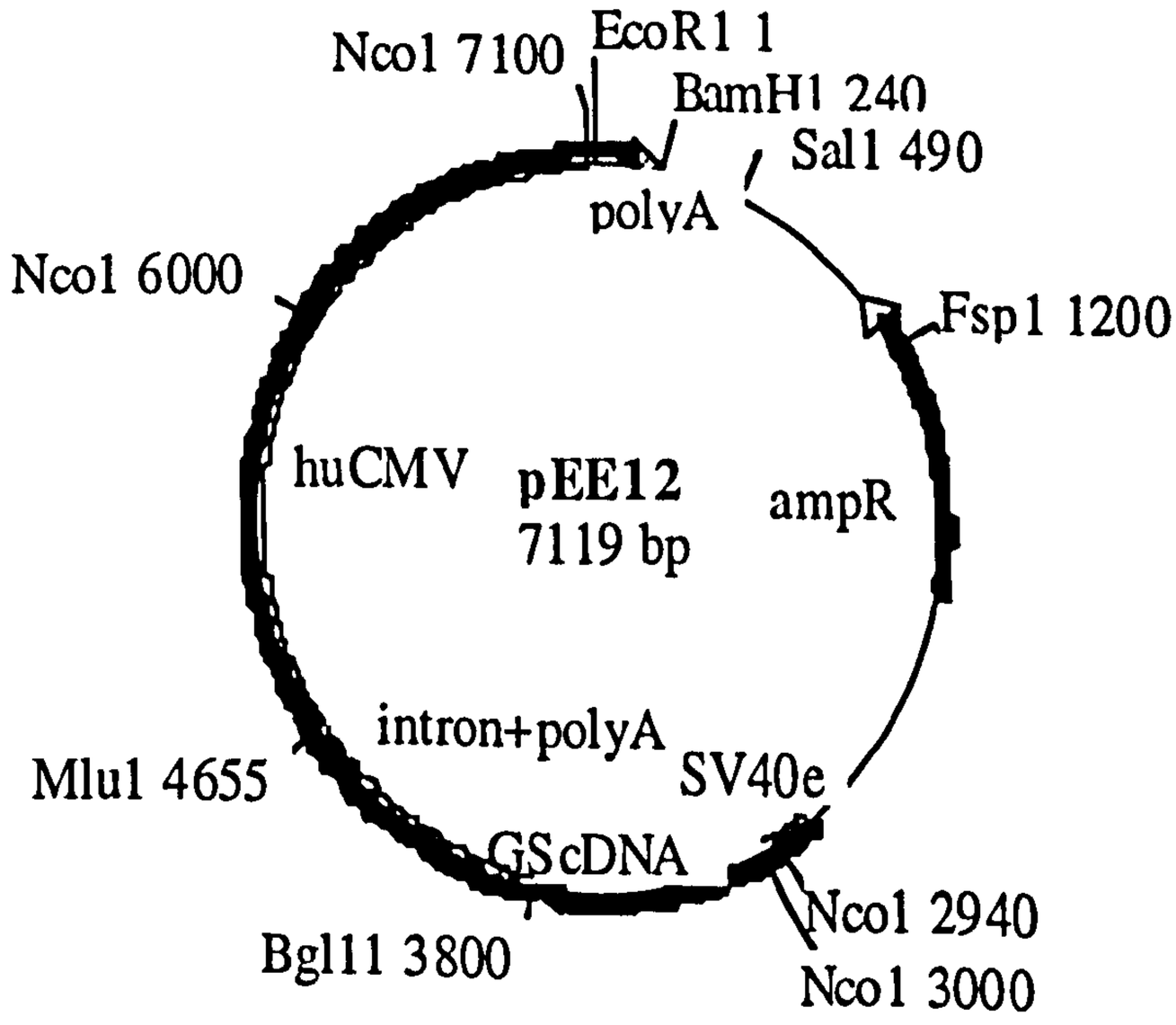
A series of experiments were designed to investigate this theory. To accomplish a full comparison of Campath-1H antibody expressed in various cellular hosts, in addition to hamster CHO and rat YO antibodies, a third expression system was utilised. The Celltech GS vector system was considered a suitable route to generate antibody levels superior to that of the CHO dhfr$^-$ system and it could also be applied to a wider range of cellular hosts (Bebbington et al 1992). The Celltech literature recommended the mouse NSO myeloma cell line, with no endogenous GS, as a suitable expression host. The expression system involves an amplification of gene copy number similar to that achieved in the CHO system. Activity of the GS gene encoded on the Celltech plasmid vectors is inhibited by the drug MSX. Low levels of MSX, in the culture medium of cells bearing the plasmid, select for cells harbouring an amplification of the GS gene plus flanking sequences and consequently an increased expression of product. To express the Campath-1H antibody in NSO myeloma cells, it was necessary to re-clone the humanised heavy and light chain cDNAs from their pAT 153-based replicons into the GS selection based vectors (Fig.4.1).

Figure 4.1 A diagrammatic representation of the Celltech Glutamine synthetase vectors a)pEE6hmcv-Bgl II and b)pEE12. Relevant restriction sites are shown. huCMV represents human cytomegalovirus major immediate early promotor, poly A represents SV40 early polyadenylation signal, SV40e represents SV40 early promotor, intron + polyA represents the small t-intron and early polyadenylation signal from SV40, GScDNA represents glutamine synthetase cDNA and ampR represents ampicillin resistance marker.

A)



B)



4.2 Aims of this chapter

This chapter reports on the cloning and transfection of the Campath-1H plasmids into NSO cells, and the derivation and selection of the transfected cell lines. The transfected cells were adapted from serum-containing (S) medium through protein-free (PF) and cholesterol-free (CF) medium to aid antibody isolation. To mimic production methods a large-scale fermentor (F) batch of antibody was also produced. Utilising the assays defined in Chapter three, the in vitro analysis and comparison of Campath-1H antibodies from different stages of medium development were recorded and changes in activity identified. N-linked glycosylation profiles for each antibody were derived and comparisons with changes in activity made.

Results

4.3 Cloning and expression of the Campath-1H cDNA in NSO myeloma cells

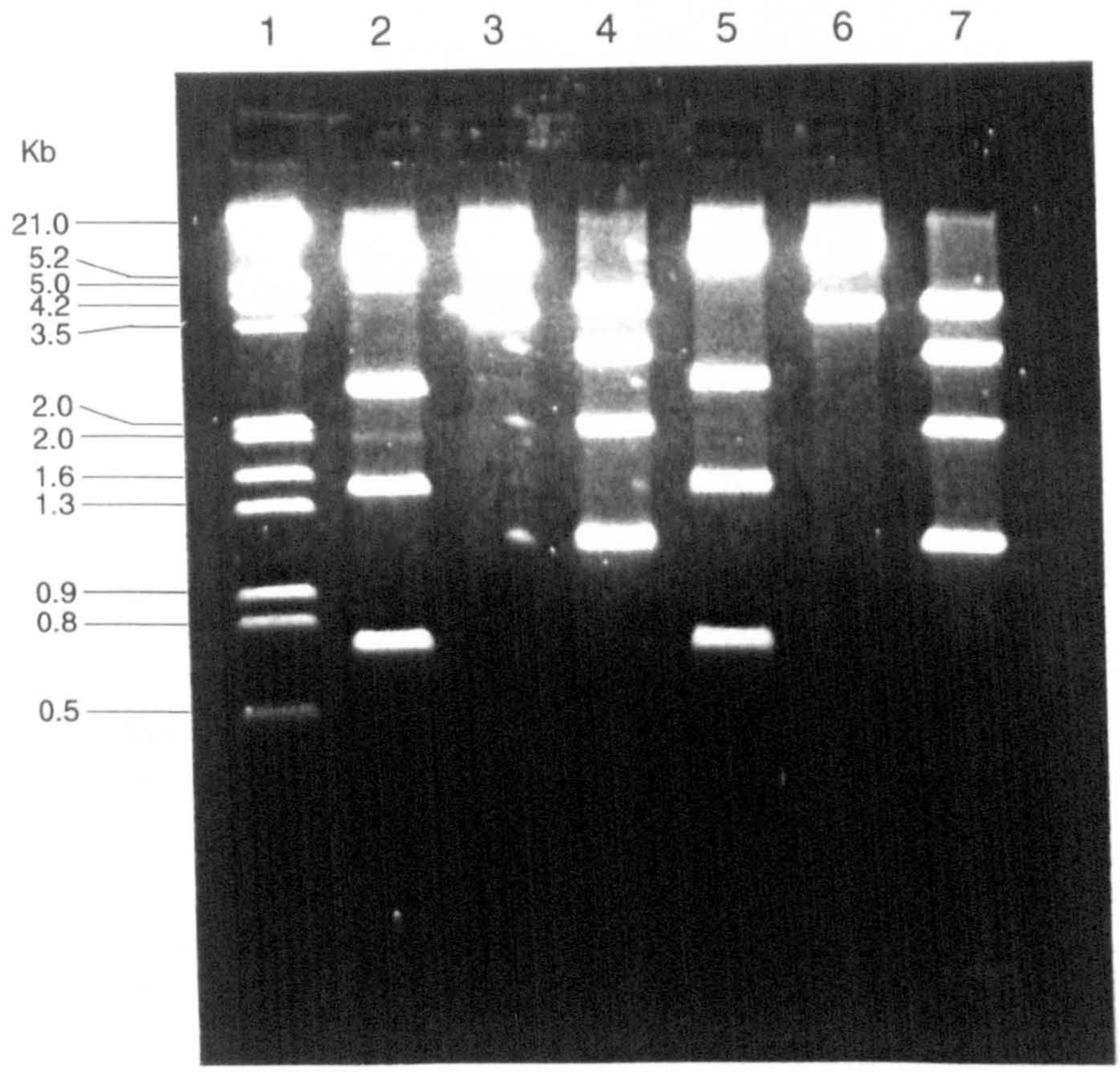
Several steps were involved in constructing and identifying NSO cells that stably directed the expression of Campath-1H antibodies under different growth conditions. The experimental results for each of these steps are outlined below .

4.3.1 The construction of GS expression plasmids encoding the heavy and light chains of Campath-1H

Two approaches were used to generate NSO cells expressing Campath-1H. In the first approach plasmids were constructed that independently encoded and directed the expression of the heavy or light chains of Campath-1H. In the second approach, the heavy and light chains were encoded on the same plasmid. For the first method, DNA encoding the Campath-1H light chain open reading frame was cloned from pL428 into the Celltech vector pEE12 by transferring a 740 bp *Hind III* DNA fragment from pL428 into *Hind III* cleaved pEE12. The orientation of the insert was confirmed by cleaving with *Bam HI*, *Nco I* and *Hind III.* One plasmid with the expected structure was identified and named pEE12+L#20. DNA encoding the Campath-1H heavy chain open reading frame was excised from pCAMPH, as a 1500 bp *Hind III* DNA fragment and ligated into the Celltech vector pEE6hcmv-*Bgl II* which had been previously cleaved with the same enzyme. A plasmid of the expected structure was identified by cleavage with *Sma I* and *Hind III* and named pEE6+H#11.

To construct a plasmid harbouring both heavy and light chains of Campath-1H along with appropriate expression sequences, pEE6+H#11 was cleaved with *Bgl I* and *Sal I* to release a 4300 bp DNA fragment which was recloned into *Bam HI* and *Sal I* cleaved pEE12+L#20. A plasmid of the correct structure was identified using *Eco RI, Nco I and Hind III* and subsequently named pEE6H12L#4 (Fig.4.2).

Figure 4.2 A gel depicting the restriction mapping of plasmid pEE6H12L#4. Lanes 1-7 are as follows; 1 *Hind III / Eco RI* digested lambda DNA markers, in duplicates 2 and 5 *Hind III* digest showing fragments at 7.4, 2.7, 1.47 and 0.7 Kb, 3 and 6 *EcoRI* digest showing bands at 7.9 and 4.2 Kb, 4 and 7 *Nco I* digest showing fragments at 4.7, 3.1, 2.3 and 1.1 Kb.



4.3.2. Expression of Campath-1H antibody in COS and NSO cells

To confirm that the GS plasmids were capable of directing the production of active antibody, DNA preparations were transfected into COS cells. Co-transfections of 5 µg each of pEE6+H#11 and pEE12+L#20, or single transfections of 10 µg of pEE6H12L#4 were utilised for transient expression. The resulting culture supernatants were analysed by FACS staining of Wien 133 cells and the antibody produced over three-five days was found to compare favourably with CHO antibody for staining (results not shown). The expression of antibody from pEE6H12L#4 was consistently more reproducible than that of the co-transfectants.

As a consequence, pEE6H12L#4 was used to construct a stable NSO cell line expressing Campath-1H. Electroporated NSO cells were dilution cloned into 96 well microtitre plates. The average number of colonies obtained at the different dilutions were 44 at

$1.67x10^4$ cells/well, 24 at $4.1x10^3$ cells/well and 7 at $8.2x10^2$ cells/well. On testing by ELISA, not all colonies were found to produce human antibody. Clones such as C7F10 which did, were progressively expanded. Specific production rate (SPR) values for first round NSO Campath-1H clones were all within the range of 1.35-1.68 µg/10^6 cells/24 hours.

To obtain higher yields of antibody, clones were exposed to medium containing a range of MSX concentrations (5-25 µM) to identify cells within the clonal populations which may have undergone spontaneous gene amplification. The cell line, C7F10 developed 6 colonies in 5 µM MSX only and none in higher concentrations. ELISA results for the colonies indicated that yields in the range of 2.5-13 µg accumulated antibody had been achieved. The Celltech guidelines estimated that a 2-10 fold increase in yield could be obtained by the use of this procedure and this appeared to be confirmed. Positive colonies such as the 5 µM MSX amplification clones of C7F10, -9D4 and -10G9 were expanded through 24 and 6 well plates before transfer to flasks. SPR values increased initially and then stabilised until assays on the cell lines gave antibody levels of 4-9 µg/10^6 cells/24 hours for -9D4 and 7-9 µg/10^6 cells/24 hours for -10G9. The amplified cells were dilution cloned and single colonies were apparent at 8 days. However, it was not until 26 days onwards that most of the colonies were sufficiently large enough to expand into 48 well plates.

The 35 colonies which appeared in this screening round were given a third series title relating to plate, row and well and supernatants were analysed for antibody content. The results were utilised to select twelve high producing clones for production purposes. Of the twelve lines selected, there was a range of accumulated antibody content between 15 and 30 µg/ml but, due to the small numbers of cells in each well, SPR values could not be estimated.

4.3.3 Calculation of SPR values for the twelve selected Campath-1H expressing clones

The twelve selected clones were expanded into 25 and 75 cm^2 flasks and multiple SPR assays performed. Initial results gave SPR values of 5.05-13 µg/10^6 cells/24 hours. This did not take into consideration the long-term growth rates of each clone and it was possible that over time the slowest growing clones could produce the highest levels of antibody. To investigate this issue further, four C7F10 clones, 9D4-4H3, 9D4-4F8, 9D4-5A11 and 10G9-8C3, were monitored for both cell number and yield changes with time.

Fresh ampoules of C7F10 10G9-8C3, 9D4-4F8, 9D4-5A11 and 9D4-4H3 were thawed from liquid nitrogen and cultured in select medium containing 5 mM MSX for 7-14 days prior to each experiment. One 25 cm^2 flask of each clone was analysed for both human antibody content and viable cell count, 24 hours post medium change and SPR values calculated. Replicate flasks were analysed 3-4 days post medium change and at other later time points (up to 28 days) and the accumulated antibody yield calculated (Table 4.1).. The resulting antibodies were not tested for biological activity in this series of experiments and so levels of degradation were not ascertained

Table 4.1 The results of monitoring specific C7F10 clones for antibody production with time. The numbers in faint type represent total yield, in µg, of the cultures. The results of three to four separate experiments are presented with the exact date of sample harvest given in parentheses. The clone 9D4-4H3 proved difficult to assay because of persistent low level bacterial contamination.

		Total antibody content (µg) at different harvest dates								
C7F10 CLONE	**Exp. No**	**D.1**	**D.3/4**	**D.6/7/8**	**D.10/11**	**D.13/14/15**	**D.18**	**D.21**	**D.25**	**D.28**
10G9-8C3	**1**	25.9	65.8(4)	146(7)	210(10)	392(13)	553	-	462	-
	2	11.9	39.9(3)	161(6)	273(11)	350(14)	539	504	630	812
	3	5.92	65.1(4)	182(8)	350(11)	434(15)	-	-	-	-
9D4-4F8	**1**	30.8	86.8(4)	158(7)	287(10)	462(13)	581	-	532	-
	2	11.9	36.4(3)	133(6)	371(11)	462(14)	511	644	392	399
	3	5.92	57.4(4)	175(8)	329(11)	567(15)	-	-	-	-
9D4-5A11	**1**	-	-	-	-	-	-	-	-	-
	2	16.1	53.2(3)	84(6)	392(11)	469(14)	581	413	721	721
	3	4.59	77(4)	315(8)	413(11)	728(15)	-	-	-	-
9D4-4H3	**1**	21.0	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-
	3a	14	32.2(4)	-	-	-	-	-	-	-
	3b	3.82	-	-	-	-	-	-	-	-

The results in Table 4.1 demonstrate variability in the individual assays when comparing antibody accumulation alone. The data is artificial in many ways in that the antibody was allowed to accumulate in "rot conditions" such that the cells were not re-fed or sub-cultured during the experiment. These are conditions not usually tolerated in production techniques, as both the cell number and degradation of antibody is uncontrolled. For comparison, the cell counts and calculated SPR values of the day one and four harvests of the same experiment are shown in Table 4.2. The results still demonstrate inconsistency for the day one samples.

Table 4.2 The SPR values of the day one and four supernatant samples from the experiment in Table 4.1. In faint type the accumulated antibody yield for each sample has been corrected for total cell count to give µg antibody /10^6 cells/time in culture. The exact date of harvest is shown in parentheses.

C7F10 Clone	Exp. no.	Day 1 Cell Count (x10^6)	Day 1 SPR values	Day 3/4 Cell Count (x10^6)	Day 3/4 SPR values
10G9-8C3	**1**	1.4	18.5	1.74	37.82(4)
	2	1.56	7.63	1.38	28.9(3)
	3	0.48	12.3	1.38	47.17(4)
9D4-4F8	**1**	0.98	31.43	1.96	44.29(4)
	2	1.44	8.27	0.92	39.6(3)
	3	0.46	12.86	1.42	40.42(4)
9D4-5A11	**1**	-	-	-	-
	2	1.88	8.56	1.86	28.6(3)
	3	0.34	13.47	2.2	35.0(4)
9D4-4H3	**1**	0.8	26.25	-	-
	2	-	-	-	-
	3a	0.84	16.67	1.28	25.16(4)
	3b	0.32	11.92	-	-

To fulfil production requirements, ampoules of the twelve cell lines were transferred to Dr. Michelle Scott, of the Biological Development Laboratories, Beckenham for further characterisation and for use as production line stocks and also to Mike Keen for the development of a custom made protein-free medium for NSO cells (Keen and Steward 1995b, Keen and Hale 1996). I continued to work with the C7F10 clones 10G9-8C3, 9D4-4F8 and 9D4-5A11.

4.3.4 Comparison of NSO clones for growth and yield in serum (S) containing or protein-free (PF) medium

The C7F10 NSO clones 10G9-8C3 and 9D4-4F8 were compared for growth and yield with time in culture in different media, (a) Celltech select medium containing serum plus 5 µM MSX; (b) a protein-free medium, WNS-B, manufactured by the Department of Media Production to Mike Keens formulation or (c) a protein-free medium, WNS-C, developed from WNS-B by Mike Keen. On days 1, 2, 3, 4 and 6, all adherent and non-adherent cells were harvested and counted and the supernatants were analysed for human antibody content by ELISA. The results are shown in Table 4.3.

Table 4.3 The growth and yield, with time, of NSO C7F10 clones 10G9-8C3 and 9D4-4F8 in various media. A total number of $1.5x10^6$ cells in 6 ml of mediums Celltech select, WNS-B or WNS-C were dispensed into flasks and their growth and antibody production were analysed on days 1, 2, 3, 4 and 6. The numbers in faint type represent either total viable cell count for each flask as multiples of 10^6 cells or total human Ig antibody yield in nanogram (ng) per flask.

	C7F10 10G9-8C3			C7F10 9D4-4F8		
	Select	**WNS-B**	**WNS-C**	**Select**	**WNS-B**	**WNS-C**
D.1 count (10^6)	0.32	0.3	0.22	0.34	0.24	0.22
yield (ng)	12.6	12.6	9.6	15	7.8	6
D.2 count (10^6)	0.5	0.42	0.32	0.4	0.34	0.2
yield (ng)	32.4	30.6	22.2	36	19.2	15.6
D.3 count (10^6)	0.86	0.5	0.4	0.56	0.5	0.6
yield (ng)	58.8	56.4	37.2	60	35.4	29.4
D.4 count (10^6)	1.08	1.06	0.7	1.52	1.12	0.96
yield (ng)	97.8	95.4	69	108.6	63	55.2
D.6 count (10^6)	0.52	0.28	0.7	1.10	0.78	1.0
yield (ng)	146.4	122.4	114	171	112.8	118.8

Although both clones grew faster and made more antibody in the Celltech media, they grew and produced antibody in WNS-B and -C. The cells in WNS-B initially grew more rapidly but began to expire by day 6. In WNS-C the initial rate of growth was slower but cell numbers did not decline. The latter would enable cells to continue producing antibody over a longer period.

At this stage in the process of selecting NSO clones for antibody production there were sufficient amounts of antibody being generated to allow functional comparisons to be made.

4.4 Comparison of NSO and CHO-derived Campath-1H antibodies

The first batch of NSO-derived Campath-1H to be purified was that from NSO 9D4-5A11 cells cultured, under laboratory conditions, in Celltech select medium containing 5% foetal calf serum (NSO.S#1). The Campath-1H isolated and purified from CHO cells for use in clinical trials is produced under serum-free conditions in fermentors. Comparisons were made between the two to confirm mode of action. In several assays

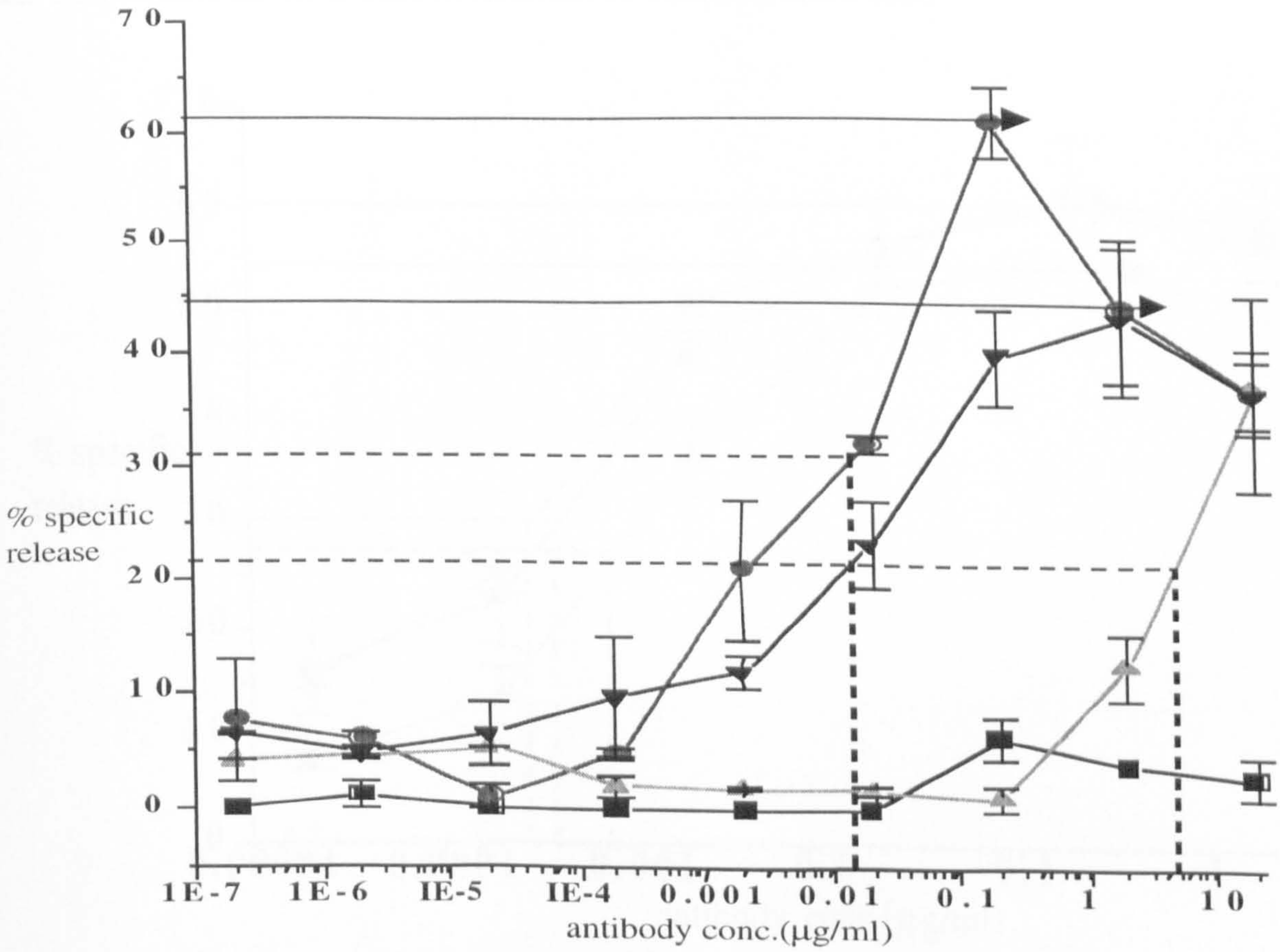
the original YO Campath antibody (donated by Dr. Geoff Hale) was also included for comparison.

4.4.1 ADCC assays

4.4.1.1 NSO-derived antibody (NSO.S) compared with clinical trial CHO Campath-1H for effector cell usage

NSO.S#1 and CHO Campath-1H antibodies were incorporated into ADCC assays in which either mononuclear or polymorphonuclear cells were used as effector cells (Fig.4.3). As with the earlier CHO experiments (Chapter three, 3.4.3) both antibodies utilised mononuclear cells preferentially and generated identical 50% of maximum lysis values.

Figure 4.3 The comparison of NSO.S and CHO Campath-1H cell type usage. CHO and NSO.S Campath-1H was compared in an ADCC in which either mononuclear or polymorphonuclear cells were utilised as effector cells at a ratio of 25:1. 10^4 Wien 133 cells were mixed with 50 µl CHO Campath plus $2.5x10^5$ polymorphonuclear effector cells (■) or CHO plus mononuclear effector cells (●), NSO Campath plus polymorphonuclear effector cells (▲) or NSO Campath with mononuclear effector cells (▼). The results are presented as percentage specific release ± standard errors. Maximum specific release values (solid horizontal lines) were calculated as 62% for CHO C1H and 44% for NSO C1H.



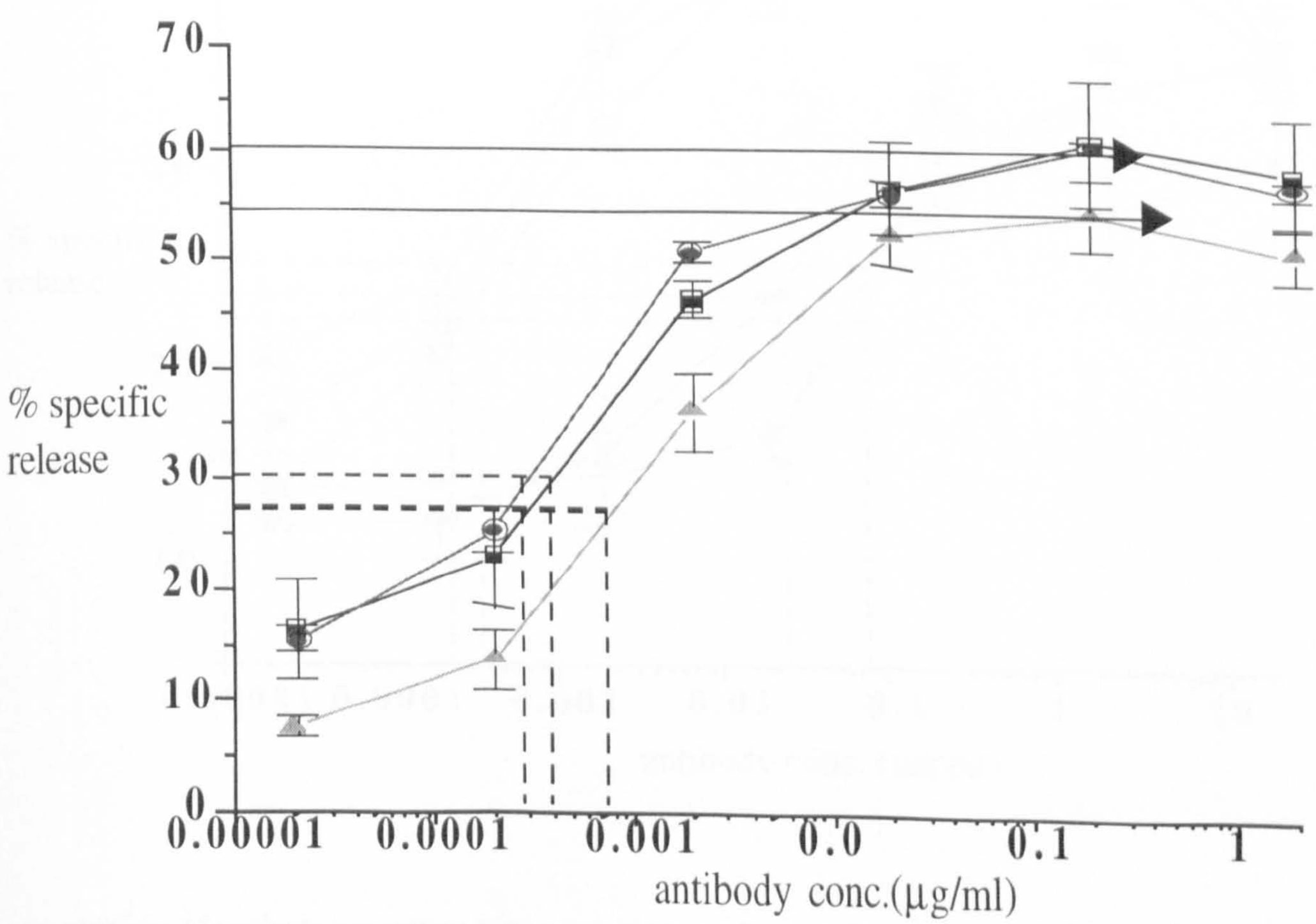
Antibody concentrations giving 50% of maximum specific release were; 0.015 µg/ml for both CHO and NSO.S C1H with mononuclear cells whilst with polymorphonuclear cells 5 µg/ml for NSO C1H and greater than 10 µg/ml CHO C1H

When a protein-free (PF) medium had been developed for NSO cells, the Campath-1H C7F10 clones 10G9-8C3, 9D4-4F8 and 9D4-5A11 were adapted to the new medium. The supernatant from the NSO clone C7F10 9D4-5A11 was purified and is referred to as NSO.PF#1. Further development of this clone involved the culture of cells in an intermediate cholesterol-free (CF) medium and finally fermentor culture (F) in protein-free media. These led to the purification of single batches of the antibodies designated NSO-CF and NSO.F respectively.

4.4.1.2 Comparison of NSO.PF, YO and CHO-derived Campath-1H.

NSO.PF#1 was incorporated into a standard ADCC assay. The assay used isolated mononuclear cells as effector cells, but had both CHO and YO antibodies as controls (Fig.4.4). To achieve 50% of maximum specific release it was necessary to utilise 0.0003 µg/ml YO antibody, 0.0004 µg/ml CHO antibody and 0.0009 µg/ml NSO.PF antibody.

Figure 4.4 The comparison of Campath-1H isolated from different cellular sources in an ADCC. CHO (■), YO (●) and NSO.PF (▲) Campath-1H was utilised in an ADCC assay in which 10^4 Wien 133 target cells were coated with Campath-1H dilutions (50 µl) prior to exposure to mononuclear effector cells at a ratio of 25:1. The results are presented as percentage release ± standard errors. Maximum specific release for CHO and YO C1H calculated as 60% and for NSO C1H 54% (solid horizontal lines).

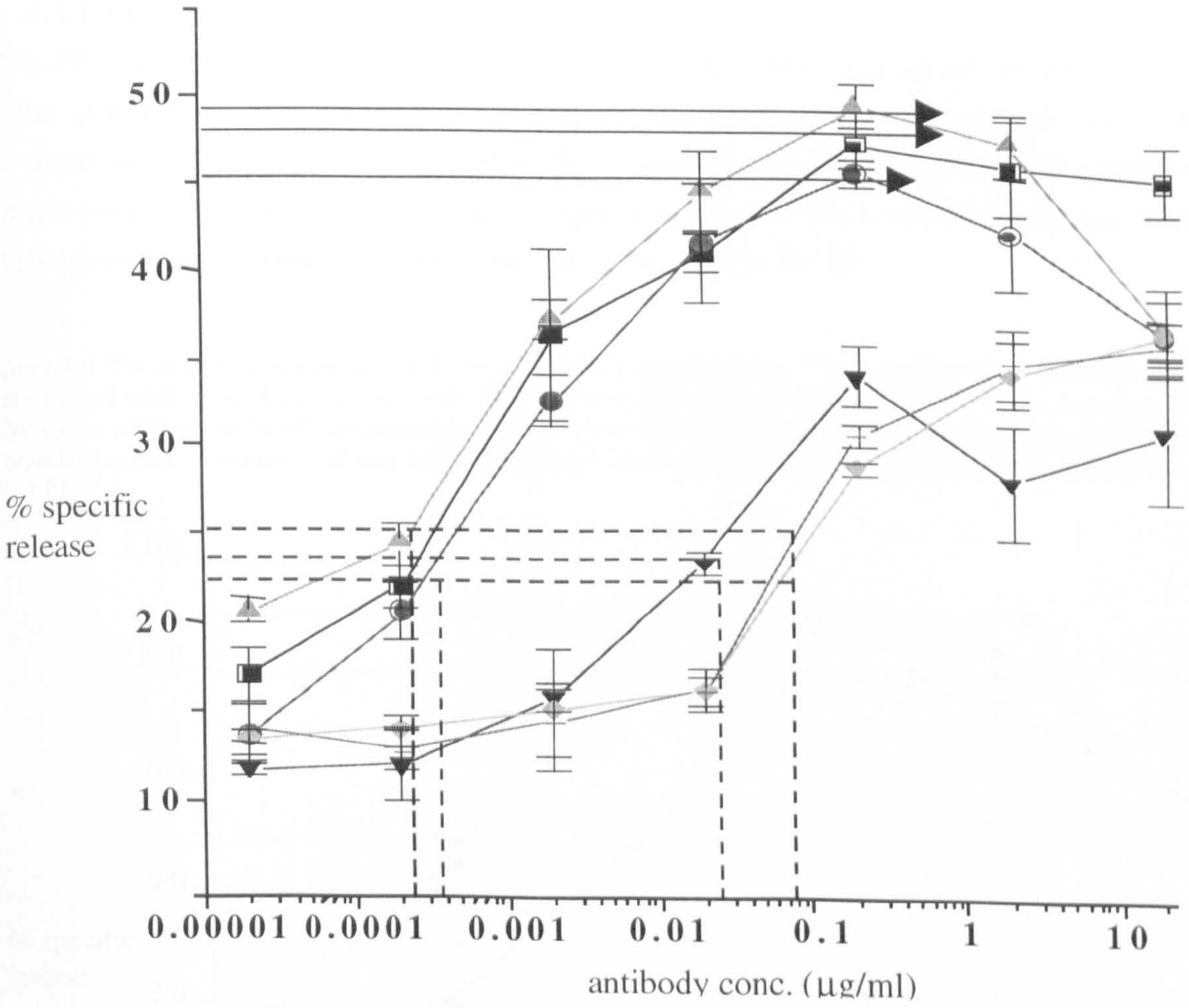


Antibody concentrations giving 50% of maximum specific release were; 0.0003 and 0.0004 µg/ml for YO and CHO C1H respectively and 0.0009 µg/ml for NSO. PF C1H.

4.4.1.3 The Ig Fc receptor usage of NSO.S and NSO. PF Campath-1H antibody in the ADCC assay.

NSO.S#1 and NSO.PF#1 Campath-1H antibodies were assessed in an ADCC assay (Fig.4.5) in which anti-Fc RIII antibody was incorporated (at a final concentration of 20 µg/ml), to block the receptor on the mononuclear effector cells. Clinical trial CHO Campath-1H was utilised as a standard and the blocking antibody was added to half of the effector cells one hour prior to their addition to the assay.

Figure 4.5 The IgG Fc receptor usage of NSO.S and NSO.PF Campath-1H antibodies in an ADCC. Wien 133 target cells (10^4), coated with 50 µl dilutions of CHO, NSO.S or NSO.PF Campath-1H, were exposed to $2.5x10^5$ mononuclear effector cells which had been pre-treated or not with anti-Fc RIII antibody. CHO antibody with untreated (■) or treated (▼) effector cells, NSO.PF with untreated (●) or treated (◆) effector cells or NSO.S with untreated (▲) or treated (✚) effector cells. The results are expressed as percentage specific release ± standard error. Maximum release values (solid horizontal lines) were calculated as 49% for NSO.S, 47% for CHO and 45% for NSO.PF.



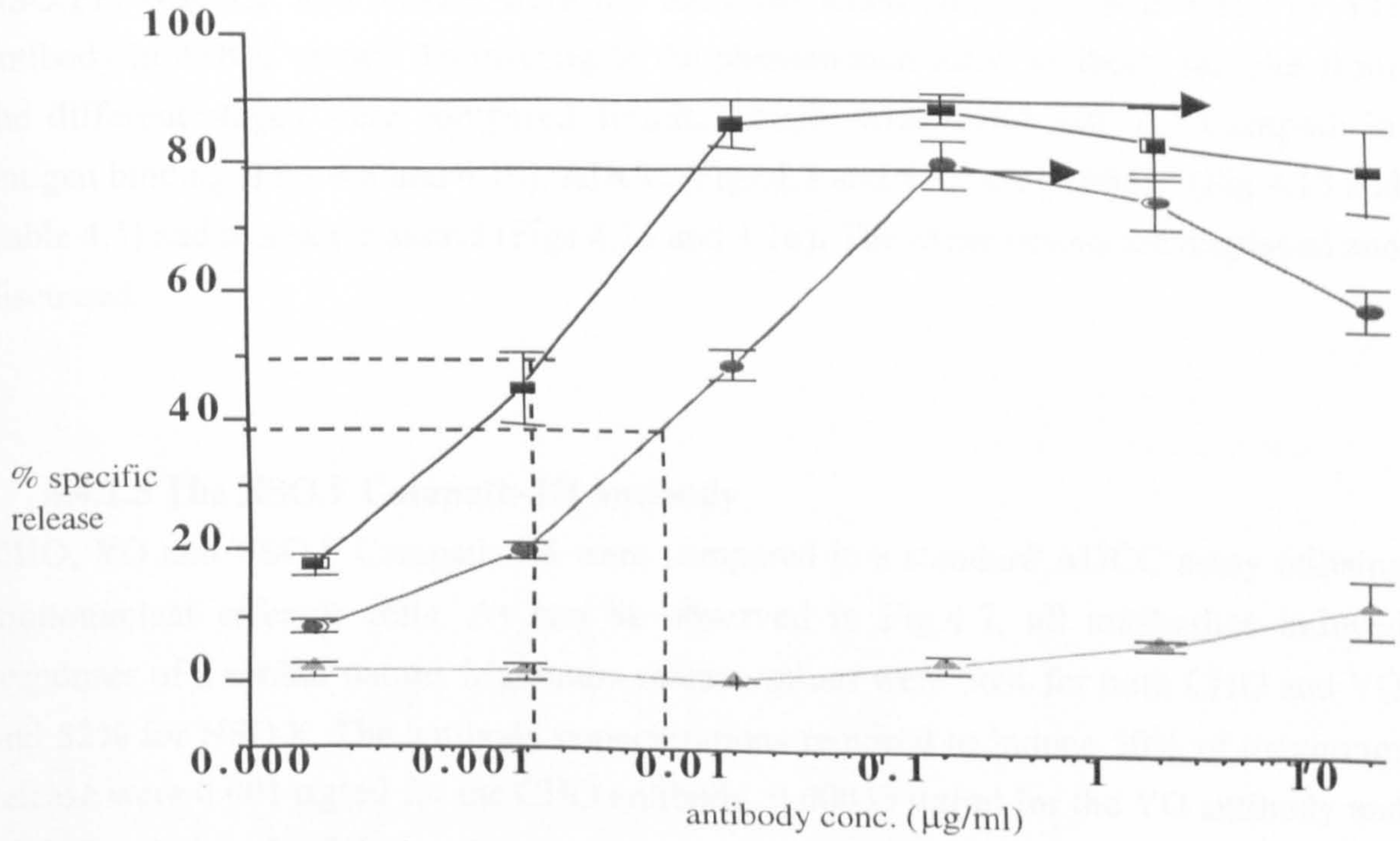
Concentrations of antibody generating 50% of maximum release in the presence of effector cells without and with anti-FcRIII treatment were; 0.00025 and 0.08 µg/ml for NSO.S respectively, 0.00025 and 0.025 µg/ml for CHO respectively and 0.00035 and 0.08 µg/ml for NSO.PF respectively.

All antibodies in the control experiment were demonstrated to engage mononuclear cells to facilitate cell lysis with little difference in efficiency over the majority of doses tested for this donor. With untreated effector cells the concentration required to give 50% of maximum specific release values for all antibodies was 0.0002-0.00035 µg/ml. The addition of anti-Fc RIII antibody to the assay influenced both NSO antibodies to the same degree at all concentrations, but CHO Campath-1H was less affected within the 0.2-0.002 range. At 2-20 µg/ml, inhibition of all antibodies was reduced. A similar result was seen in earlier CHO antibody ADCC experiments (Chapter three, section 3.4.4) and was thought to be a result of insufficient blocking of the Fc RIII receptor at high doses of Campath-1H antibody. As with the CHO experiments, the anti-Fc R antibodies were never tested independently and thus not shown to influence receptor function.

4.4.1.4 Effect of NSO.PF antibody deglycosylation

NSO.PF#1 antibody was deglycosylated enzymatically with N-glycanase by Anne Lines of the Department of Biological Development, Wellcome Research Laboratories. Both the intact and deglycosylated NSO antibodies were utilised in a standard ADCC assay, in comparison with intact CHO Campath. Deglycosylation of the CHO antibody had been shown previously to ablate activity (Chapter three, section 3.4.5).

Figure 4.6 The effects of removing the NSO.PF antibody carbohydrate. ^{51}Cr-Labelled Wien 133 cells (10^4) were mixed with 50 µl dilutions of CHO (■), NSO.PF (●) or deglycosylated NSO.PF(▲) Campath-1H prior to the addition of $2x10^5$ mononuclear effector cells. Results are expressed as mean % specific release ± standard errors. Maximum release (solid horizontal line) was calculated at 90% for CHO, and 79% for NSO.PF.



Antibody concentration generating 50% of maximum release was calculated as 0.0021 µg/ml for CHO and 0.009 µg/ml for NSO.PF. Deglycosylation of NSO.PF ablated activity as seen for CHO-C1H.

Fig.4.6 demonstrates that, with this donor, the intact NSO.PF#1 antibody mediated ADCC less efficiently than CHO antibody at all dilutions under investigation. The concentration required to give 50% of maximum specific release was 0.0017 µg/ml for the CHO antibody whilst it increased to 0.009 µg/ml for the NSO.PF antibody. Deglycosylation of the NSO.PF antibody totally ablated ADCC activity at all doses.

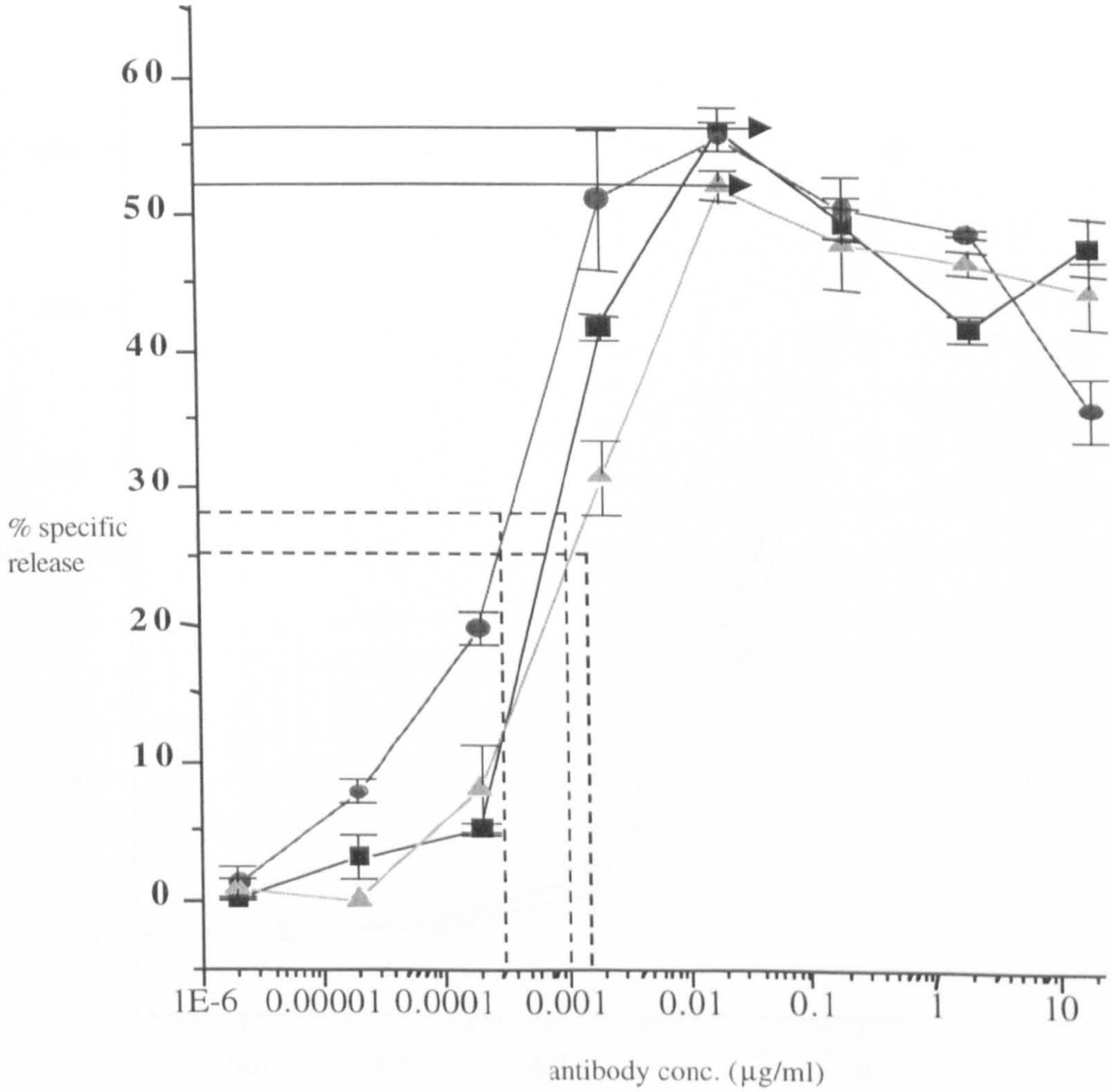
After trial in an experimental cholesterol-free medium, C7F10 9D4-5A11 was the clone chosen for development in a small 2 litre fermentor. It was placed into production, in protein-free medium, by Dr. Michelle Scott of the Department of Biological Development, Wellcome Research Laboratories, Beckenham, Kent. The antibody was produced over a limited period in the absence of feeding and the cells were regularly monitored for viability. Yields from day 4 and day 6 culture supernatants were analysed and found to contain 11 µg/ml and 44 µg/ml human antibody respectively (Michelle Scott, personal communication). This was not unexpected in that prior analysis had shown that antibody was maximally produced when the cells were not dividing rapidly. The antibody (NSO.F) was purified in a manner similar to the CHO clinical trial antibody and was compared in assays with CHO Campath-1H, previous forms of NSO antibodies and YO Campath-1H produced in Cambridge.

The chronological analysis (see 2.4.9 and 2.4.10 for dates of production) of NSO antibody produced from different stages of media development suggested that NSO.S, NSO.PF, NSO.CF and NSO.F were not identical when compared with CHO or YO antibody in ADCC assays. To investigate the phenomenon fully, antibody samples from the different stages were compared simultaneously with CHO and YO Campath in antigen binding (Figs 4.8 and 4.10), ADCC (Figs.4.7 and 4.9), crosslinking (Fig 4.13 and Table 4.5) and monocyte assays (Figs 4.15 and 4.16). The observations are displayed and discussed.

4.4.1.5 The NSO.F Campath-1H antibody

CHO, YO and NSO.F Campath-1H were compared in a standard ADCC assay utilising mononuclear effector cells. As can be observed in Fig.4.7, all antibodies induced responses of a similar nature. Maximum release values were 56% for both CHO and YO and 52% for NSO.F. The antibody concentrations required to induce 50% of maximum release were 0.001 µg/ml for the CHO antibody, 0.00035 µg/ml for the YO antibody and 0.0015 µg/ml for NSO.F.

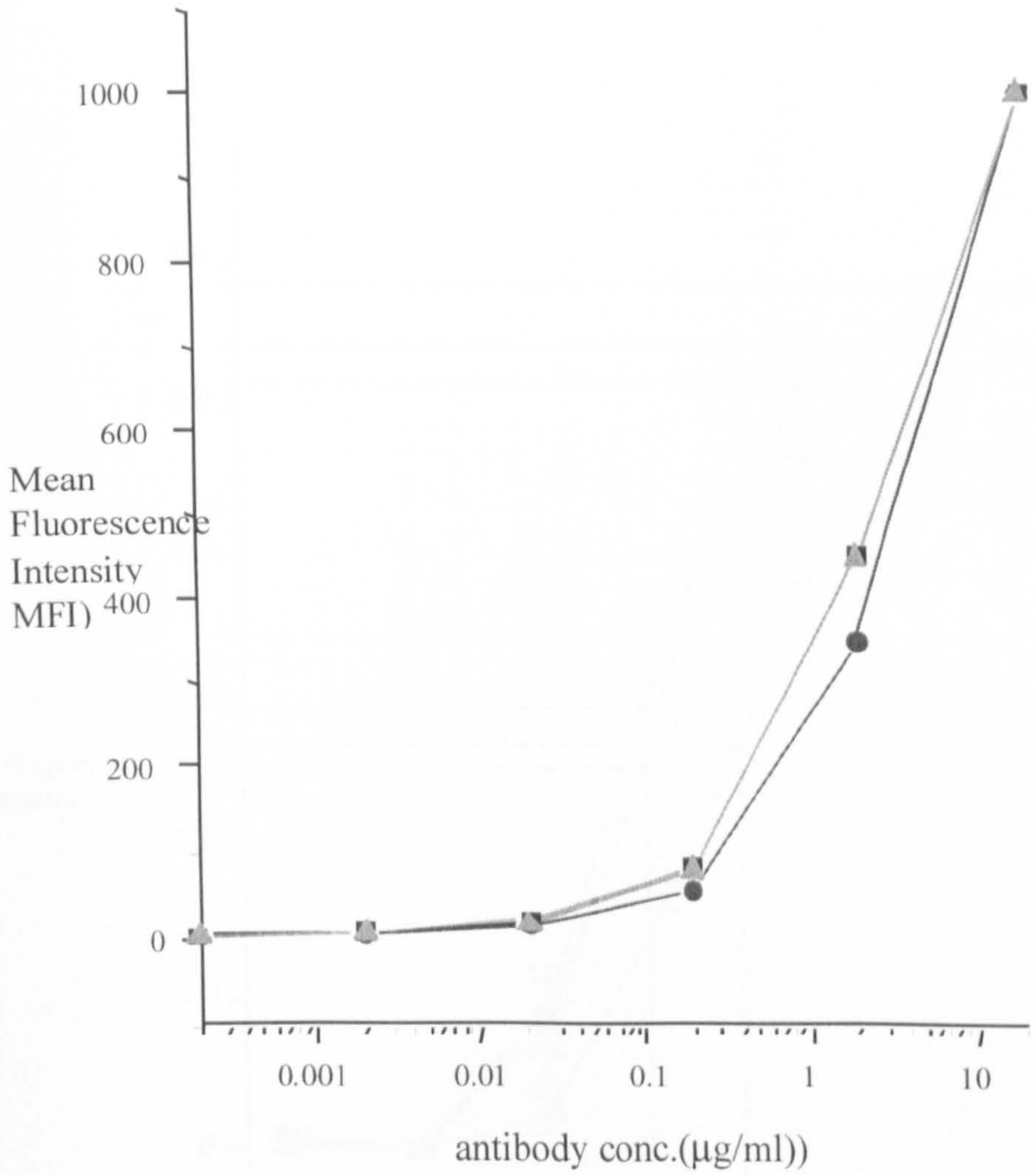
Figure 4.7 A comparative Campath-1H ADCC. Campath antibody dilutions (50 µl) were mixed with 10^4 ^{51}Cr labelled Wien 133 cells prior to the addition of $2x10^5$ mononuclear effector cells. The centrifuged supernatants were assessed for Chromium release five hours later. CHO (■),YO (●) and NSO.F (▲) Campath-1H. The results are displayed as mean % specific release ± standard errors. Maximum release was taken as 56% for YO and CHO whilst NSO.F was 52%.



Antibody concentrations of 0.00035, 0.001 and 0.0015 µg/ml (YO, CHO and NSO.F respectively) were required to induce 50% of maximum release.

The corresponding antibody/antigen binding data for these antibodies is shown in Figure 4.8. The antibodies were serially diluted from 20 µg/ml and mixed with antigen expressing cells as described in Materials and Methods. They demonstrated similar antigen binding at concentrations able to mediate ADCC.

Figure 4.8 The mean fluorescence intensity of dilutions of Campath antibodies from different sources. Aliquots of antibody (50µl) ● YO, ▲ NSO.F and ■ CHO were mixed with Wien 133 cells ($5x10^4$) for 30 minutes prior to detection with anti-human IgG-FITC. Samples were fixed and analysed on a Becton Dickinson FACS analyser.

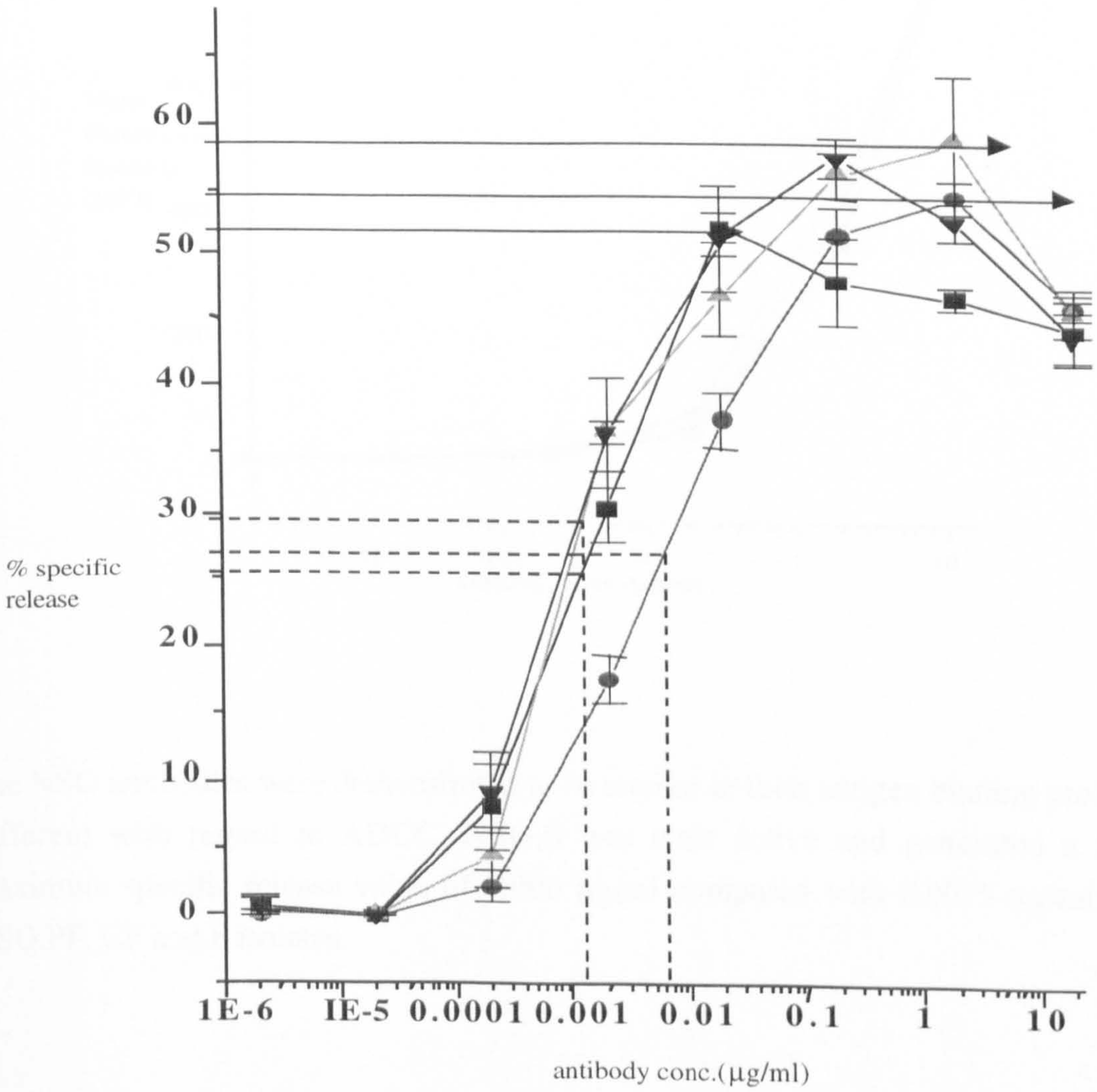


4.4.1.6 Comparison of NSO antibodies produced from cells grown under different conditions

Fig.4.9 demonstrates the results of comparing NSO.S#1, NSO.PF#2, NSO.CF and NSO.F, for clarity the data for CHO and YO antibody are not included (see Fig. 4.7 for the same experiment). The antibodies generated similar profiles except for NSO.S which required 0.006 µg/ml to achieve 50% of maximum lysis compared to 0.0015 µg/ml for the others. Prior to the assay, antibody concentration and binding efficiency were

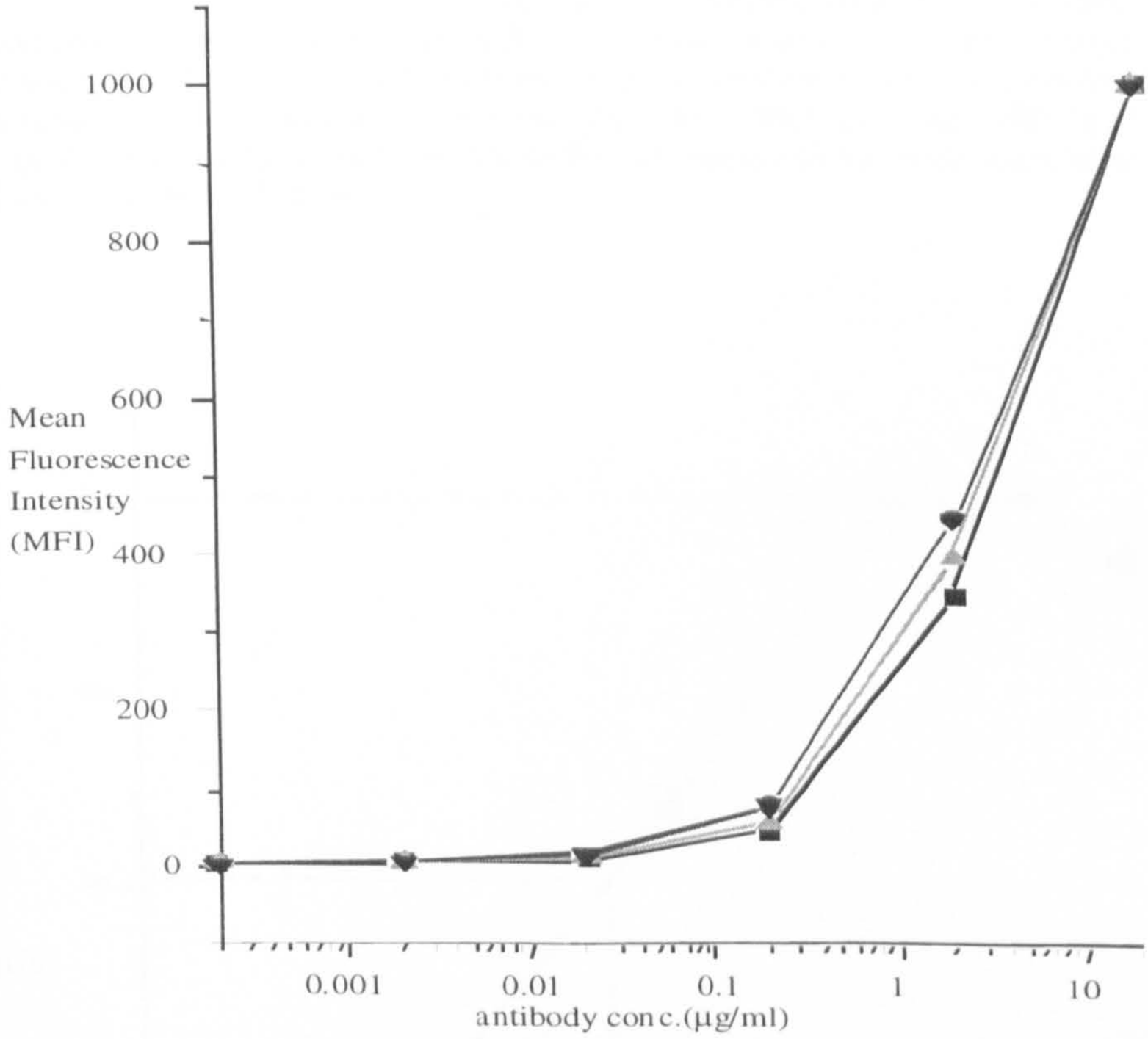
determined by anti-human Ig ELISA (data not shown) and FACS analysis (Fig 4.10), respectively.

Figure 4.9 A Campath ADCC comparing different NSO antibodies. Dilutions of NSO antibodies (50 µl) were incubated with 10^4 labelled Wien 133 target cells prior to the addition of $2x10^5$ mononuclear target cells. Chromium release was measured after five hours. Campath-1H antibodies NSO.S (●), NSO.PF (▲), NSO.CF (▼) and NSO.F (■) are compared. The results are given as mean percentage specific release ± standard errors. Maximum release values (solid horizontal lines) were 58% for NSO.PF and NSO.CF, 54% for NSO.S and 52% for NSO.F.



Antibody concentration required to generate 50% of maximum release was 0.0015 µg/ml for NSO.PF, CF and F. NSO.S required 0.006 µg/ml.

Figure 4.10. The mean fluorescence intensity of dilutions of NSO.S, .CF, .PF and NSO.F Campath antibodies. Aliquots of antibody (50µl) ■ for NSO.S, ● NSO.PF, ▲ for NSO.CF and ▼ for NSO.F were mixed with Wien 133 cells ($5x10^4$) for 30 minutes prior to detection with anti-human IgG-FITC. Samples were fixed and analysed on a Becton Dickinson FACS analyser.



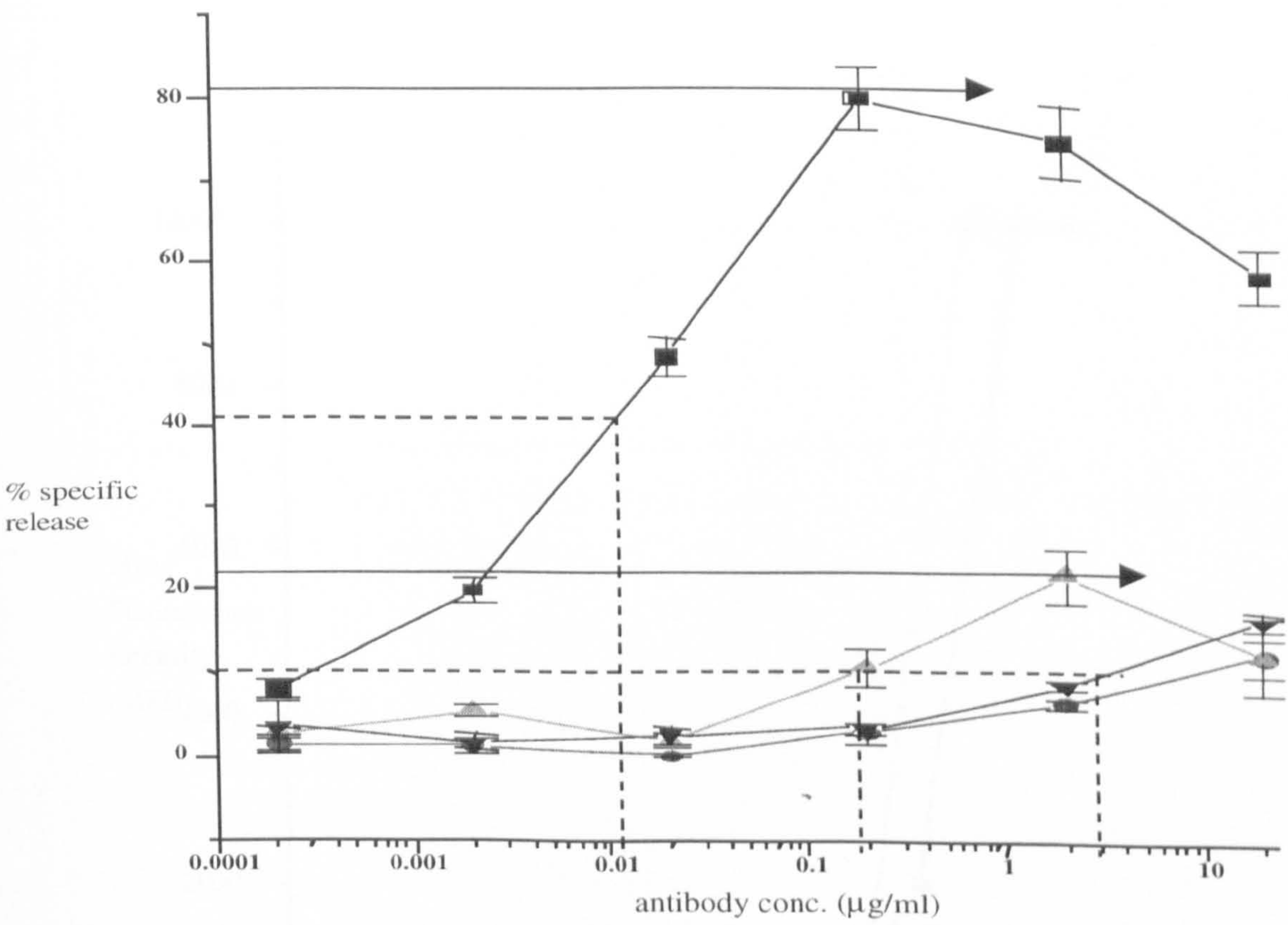
The NSO antibodies were demonstrated to be similar in their antigen binding profiles but different with regard to ADCC. NSO.S was least active and generated a 50% of maximum specific release value of 0.006 µg/ml compared with 0.0015 µg/ml for the NSO.PF, CF and F isolates.

4.4.1.7 IgG4 compared with intact and deglycosylated IgG1

NSO Campath-1H of the IgG4 isotype was produced at Burrough's Wellcome Laboratories, Research Triangle Park, North Carolina, USA. The cell line was sent to England for adaptation to protein-free medium and comparison with the NSO.PF IgG1 antibody. The isotype IgG4 was not thought to bind IgG Fc RIII in vitro and as such should not activate ADCC.

On incorporation into ADCC assays the intact NSO IgG4 subclass was found to be less able than NSO.PF#1 IgG1 to mediate specific lysis of coated targets (Fig.4.11). 50% of maximum specific release was achieved with 0.18 µg/ml (IgG4) compared to 0.012 µg/ml (IgG1). Deglycosylation of the antibodies ablated both IgG1 and IgG4 specific lysis at most concentrations.

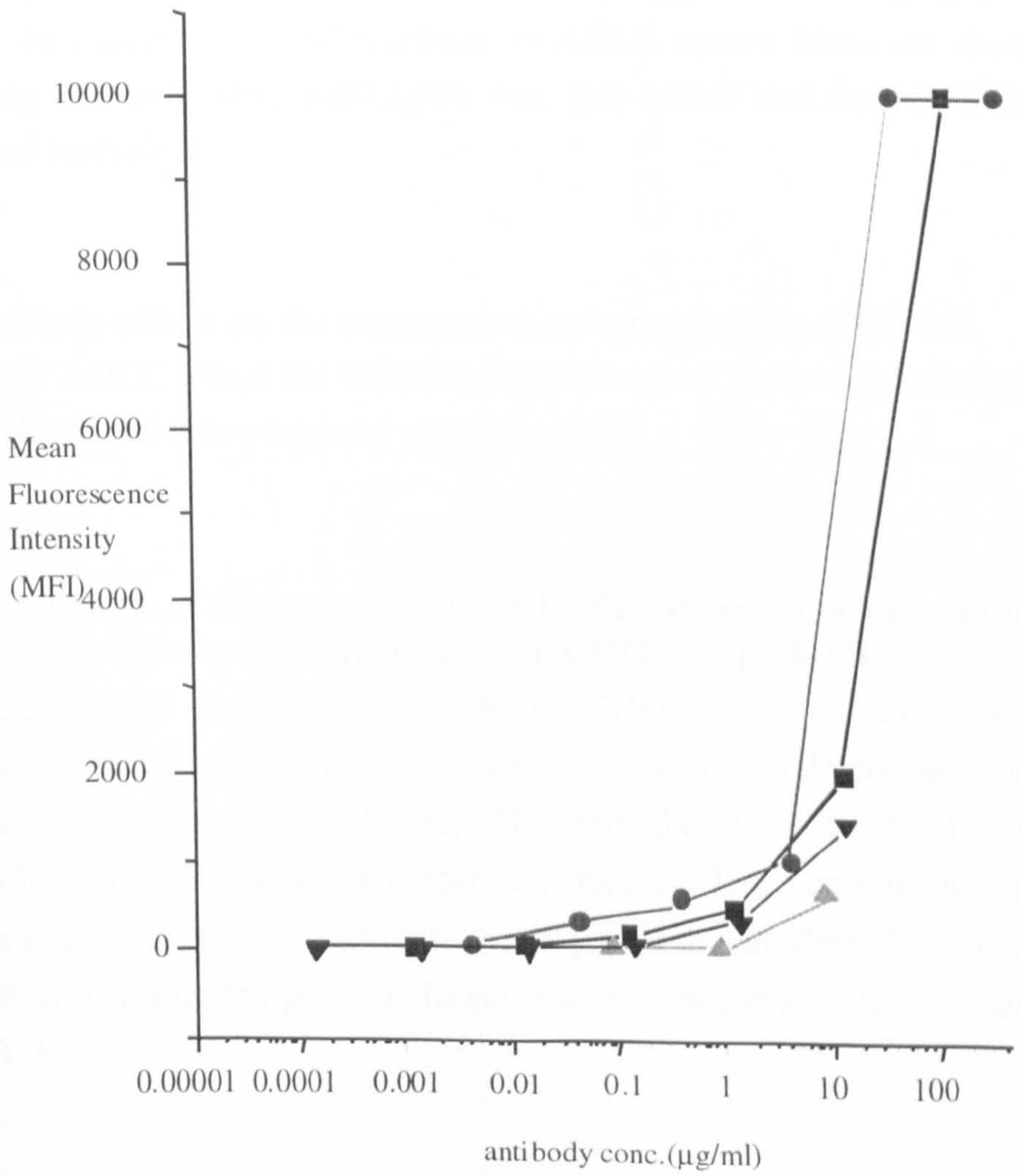
Figure 4.11 A comparison of IgG1 and IgG4 isotypes in a Campath-1H ADCC. Dilutions (50 µl) of intact or deglycosylated NSO IgG1 and IgG4 antibodies were mixed with 10^4 ^{51}Cr labelled target cells 1 hour prior to the addition of $2x10^5$ mononuclear effector cells. Chromium release was monitored five hours later. The data are expressed as % specific release ± standard errors. NSO IgG1 intact (■), IgG1 deglycosylated (●), NSO IgG4 intact (▲), IgG4 deglycosylated (▼). Maximum release values were taken as 81% for NSO.IgG1 and 22% for NSO.IgG4.



Antibody concentrations inducing 50% of maximum release were 0.012 µg/ml for NSO.IgG1 and 0.18 µg/ml for NSO.IgG4. Deglycosylation of both antibodies led to a requirement of greater than 3 µg/ml.

The NSO antibodies used in the ADCC assay (IgG1 1.26, IgG4 4.0 and deglycosylated IgG4 0.14 mg/ml, respectively) were diluted 1 in 10 from neat and then serially diluted stepwise ten fold. The dilutions were checked for antigen binding as in the Material and Methods section. The data is shown (in Fig 4.12) and indicates that, irrespective of differing starting concentration, the binding slopes were similar except for deglycosylated NSO.PF IgG1 which, after preparation, was very dilute (0.084 mg/ml).

Figure 4.12. The Mean fluorescence intensity values of intact or deglycosylated NSO.PF IgG1 and NSO.IgG4. Aliquots of antibody (50µl) ■ NSO.PF IgG1, ● NSO.IgG4, ▲ deglycosylated NSO.PF IgG1 and ▼ deglycosylated NSO.IgG4 were mixed with Wien 133 cells ($5x10^4$) for 30 minutes prior to detection with anti-human IgG-FITC. Samples were fixed and analysed on a Becton Dickinson FACS analyser.



4.4.2 Aggregate content of the NSO.PF and YO antibodies

Both the NSO.PF#1 and YO antibodies utilised in the previous experiments had been produced on relatively small scales. NSO.F and CHO Campath-1H had been manufactured by a large scale production method which involved purification of the monomeric form only. On testing, the CHO material had been demonstrated to contain 1.1% aggregate by HPLC (Chapter three, section 3.3.1).

To determine the levels of aggregate present in the YO, NSO.PF and NSO.F samples, aliquots of the antibodies were sent for size exclusion HPLC aggregate analysis, to Dr. Mark Easton of the Department of Biological Development. The C7F10 9D4-5A11 NSO.PF#1 material was found to contain 1.75% aggregate, whilst the NSO.F contained 1.2% and the YO material had a surprising 22.93%. It was postulated that this large amount may have formed due to storage at -20^{o}C and was perhaps the reason for the antibodies superior performance in ADCC assays. To verify the result, Dr. Geoff Hale provided a freshly prepared sample of the YO antibody which was found to contain less than 1% aggregate. This was compared with the original YO Campath-1H and was still found to be superior to CHO antibody in ADCC assays (data not shown) therefore disproving this postulate. NSO.IgG4 was also tested and found to contain 10.4% aggregated antibody.

4.4.3 Antibody effects on the engagement and crosslinking of CDw52

As with the ADCC assays the different antibodies were analysed chronologically as they were produced and then compared simultaneously.

4.4.3.1 Effects of the engagement of CDw52 antigen on target cells by NSO.PF and YO antibodies in comparison with CHO Campath-1H

Experiments were designed in which the CHO, YO and NSO.PF#2 Campath-1H antibodies were assessed in antigen crosslinking assays to determine whether levels of aggregation altered biological efficacy. The antibodies were tested at two concentrations selected from the CHO data in Chapter three (section 3.5.2) and the 20 µg/ml results are illustrated in Table 4.4 overleaf (data for 40 µg/ml are similar). The target cells utilised for these assays were the parental Jurkat line and the cloned CDw52 transfected Jurkat line, 3/B11.

The table shows that Campath antibodies added to the parental Jurkat cell line had little effect on their thymidine uptake (control 801,873 cpm reduced by between 56,500 to

37,200 cpm) whilst anti-human IgG and anti-HLA reduced the thymidine incorporation of parental cells by approximately 30,000 cpm. Crosslinking of any of the Campath antibodies with anti-human IgG gave incorporation values similar to anti-human IgG alone. When CHO and YO antibodies were added to CDw52 transfected Jurkat 3B/11 cells (control 763,662 cpm), they reduced thymidine incorporation by approximately 80,000 cpm and 17,000 cpm, respectively. The NSO.PF#2 antibody reduced the uptake by almost 160,000 cpm. In combination with anti-human IgG, YO, CHO and NSO.PF Campath-1H all gave similar results (40,000 cpm). From the data it can be deduced that NSO.PF (minus anti-human IgG) is not toxic to Jurkat cells as only the transfected 3/B11cells are affected, but suggests that NSO.PF is capable of inhibiting the growth of 3/B11 cells as a result of binding the antigen in the absence of crosslinking. The effect is not a consequence of aggregate content because the YO material (22.3% aggregate) demonstrates less inhibition than both NSO.PF (1.75% aggregate) and CHO (less than 2%) antibodies. However, by substituting CHO monomeric Campath-1H with antibody containing 80% aggregate it is possible to achieve identical results to that of NSO.PF in the absence of crosslinking (see Chapter three section 3.5.3).

Table 4.4. The effects of crosslinking CDw52 with intact and deglycosylated (deglyco) Campath-1H antibody from different cellular sources. Parental or transfected Jurkat cells were mixed with either 100 µl 20 µg/ml Campath-1H antibody or 50 µl 20 µg/ml Campath-1H antibody plus 50 µl anti-human IgG (HuIg) at the same concentration. Thymidine incorporation was measured at day five. The results are expressed as both mean cpm and % control value and are typical of several assays.

	Thymidine incorporation			
Antibody used	**Jurkat cells**		**Jurkat + CDw52, 3/B11 cells**	
	cpm	**%control**	**cpm**	**%control**
control	801873.3	100%	763662.9	100%
CHO	745331.8	92.9%	685252.3	89.7%
YO	764655.8	95.4%	746310.4	97.7%
NSO.PF	756188.0	94.3%	604817.5	79.2%
Deglyco. NSO.PF	728229.7	90.8%	673888.9	88.2%
a-HLA	774615.1	96.6%	722426.5	94.6%
HuIg	777165.3	96.9%	737207.5	96.5%
CHO+HuIg	768236.2	95.8%	404831.9	53.0%
YO+HuIg	779220.1	97.2%	394105.7	51.6%
NSO.PF+HuIg	792815.8	98.9%	383879.4	50.3%
Deglyco.NSO+HuIg	793179.5	98.9%	395276.9	51.8%
a-HLA+HuIg	798878.8	99.6%	756034.2	99.0%

Dilution of the same antibodies in dose response curves over the range 90-0.1 µg/ml (data not presented) demonstrated that crosslinked YO antibody, became less efficient at inhibiting thymidine incorporation once diluted below 10 µg/ml whilst NSO.PF was effective even at 1 µg/ml.

4.4.3.2 Does deglycosylated NSO.PF antibody induce cytostasis?

Deglycosylated NSO.PF was used in assays in comparison with intact NSO.PF#2 plus CHO, YO and anti-HLA controls. The inclusion of the latter antibody was to demonstrate antigen specificity. When the antibodies were incorporated into the assay at 20 µg/ml, the results tabulated in Table 4.4 were obtained.

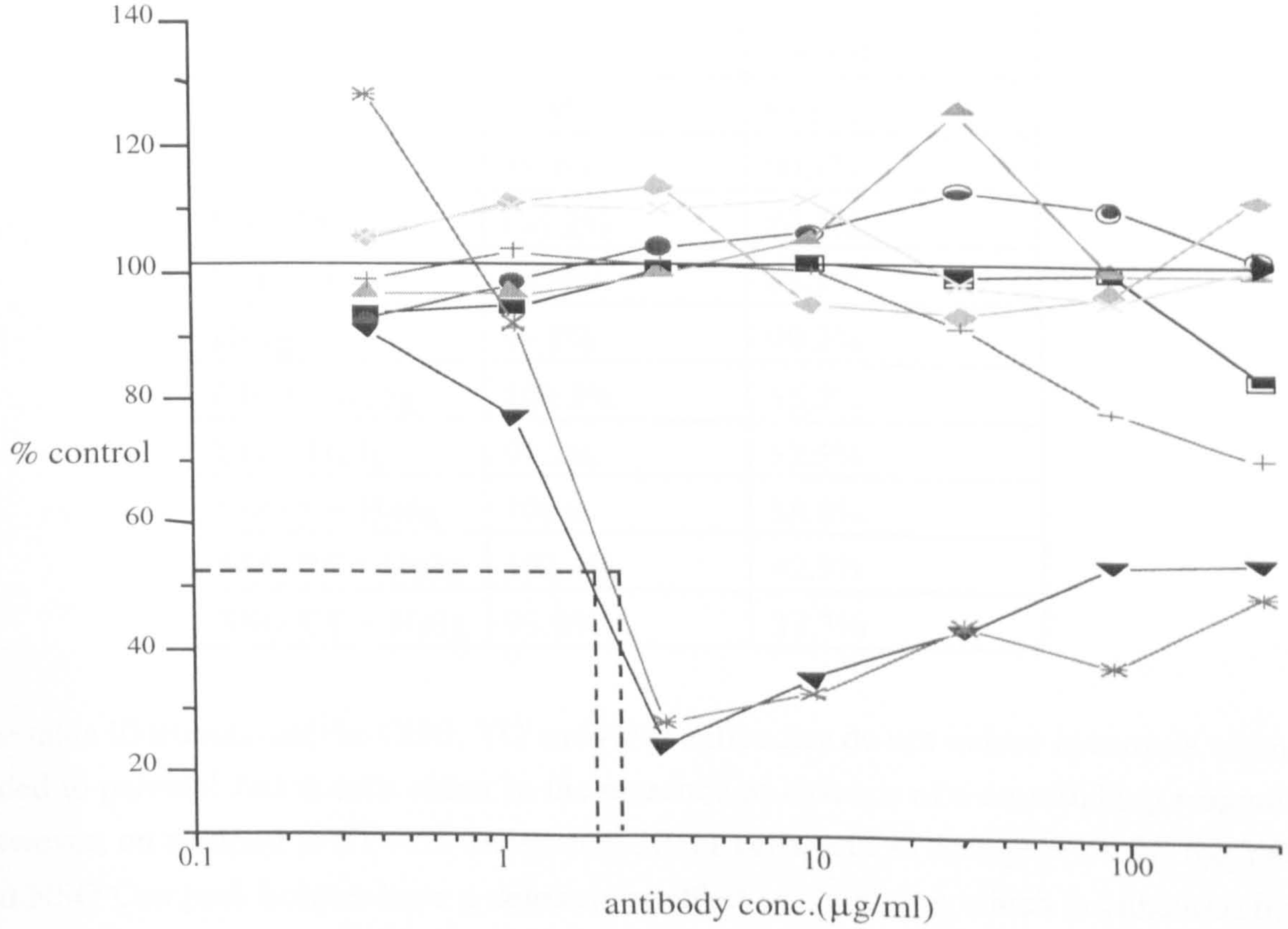
The data indicate that carbohydrate is not essential to inhibit thymidine incorporation. All of the antibodies in this assay are demonstrated to be of comparable activity and the inclusion of the anti-HLA antibody confirms that the reaction is CDw52 antigen specific.

4.4.3.3 NSO.F and CHO Campath crosslinking abilities are compared

Jurkat and transfected Jurkat 3/B11 cells were utilised as target cells in assays in which NSO.F and CHO antibodies (270-0.37 µg/ml, in the presence or absence of anti-human IgG), were compared for their ability to inhibit thymidine incorporation (Fig.4.13).

If crosslinked with anti-IgG, both NSO.F and CHO Campath-1H were able to inhibit the thymidine incorporation of CDw52 transfected Jurkat cells over the range 270-3 µg/ml, with maximum inhibition at 3 µg/ml. The graduated loss of inhibition observed at concentrations greater than 3 µg/ml may indicate that insufficient cross-linking antibody was present. CHO antibody was still able to exert a 20% inhibition on the transfected cells at 1 µg/ml, whilst NSO.F Campath was not. Values for the concentration of antibody required to achieve 50% inhibition are 2 and 2.5 µg/ml for the CHO and NSO.F antibodies respectively. In the absence of crosslinking, both antibodies reduced the thymidine incorporation of the transfected cells at doses greater than 30 µg/ml.

Figure 4.13 The comparison of NSO.F Campath-1H with CHO antibody for engagement and crosslinking abilities. CHO or NSO.F Campath-1H was added to either Jurkat or CDw52 transfected Jurkat cells in the presence or absence of 50 µl anti-human IgG at 20 µg/ml. Thymidine incorporation was monitored after five days in culture. CHO antibody with Jurkat cells, plus (▲) or minus (■) anti-human IgG, CHO antibody with transfected Jurkat cells plus (▼) or minus (●) anti-human IgG, NSO antibody with Jurkat cells plus (✖) or minus (◆) anti-human IgG, NSO antibody with transfected Jurkat cells plus (✳) or minus (✚) anti-human IgG. The results are presented as % control value with no standard errors and are representative of several assays. Maximum thymidine incorporation (solid horizontal line) is 100% control.



CHO and NSO antibody concentrations required to achieve 50% inhibition were 2 and 2.5 µg/ml respectively.

4.4.3.4 Comparison of NSO isolates ability to engage and crosslink antigen

NSO.S#1, NSO.PF#2 and NSO.CF were compared with CHO and YO Campath to establish whether culture conditions and/or medium could alter their ability to bind to antigen. The antibodies were added to parental or transfected 3B/11 cells in the presence or absence of anti-human IgG antibody and the data is presented overleaf in Table 4.5.

Table 4.5 Comparing the ability of NSO antibody isolates to engage and crosslink antigen. Aliquots of 100 µl or 50 µl 20 µg/ml NSO antibody were incubated with 100 µl parental or CDw52 transfected 3B/11 cells (10^5/ml) in the presence or absence of 50 µl 20 µg/ml anti-human IgG (HuIg). The data is presented as percentage control value for each antibody and cell type.

	% Thymidine incorporation	
Antibody used	**Jurkat**	**Jurkat+ CDw52**
control	100%	100%
CHO	100.5%	100.6%
YO	99.8%	83.6%
NSO.S	98.9%	90.1%
NSO.PF	101.2%	82.7%
NSO.CF	95.5%	65.3%
HuIg	99.5%	99.2%
CHO + HuIg	100.3%	55.7%
YO + HuIg	97.2%	52.5%
NSO.S + HuIg	100%	38.8%
NSO.PF + HuIg	102.4%	42.9%
NSO.CF + HuIg	93.8%	37.3%

The table illustrates that the CHO, YO and NSO antibodies do not induce cytostasis when added to parental Jurkat cells either in the presence or absence of a crosslinking reagent. However, on addition to CDw52 transfected 3B/11 cells, and as seen previously, the YO and NSO Campath isolates have a deleterious effect on their own which is enhanced by the introduction of anti-human IgG. The ability of the crosslinked CHO and YO antibodies to induce cytostasis is less than that of the various NSO antibodies. The NSO antibodies were not demonstrated to bind antigen to different extents by FACS analysis (see Fig.4.8) and thus the observed differences may be entirely due to levels of specific toxicity for the transfected cells.

4.4.3.5 Engagement and crosslinking abilities of IgG4 Campath compared with IgG1

NSO.PF#1 IgG1 and IgG4 antibodies were placed into assays in which parental or transfected 3B/11 target cells were present and anti-human IgG was utilised as a crosslinking agent. The results are tabulated in Table 4.6 overleaf.

Table 4.6 A comparison of the engagement and crosslinking abilities of NSO IgG1 and IgG4 isotypes. A summary of the % control values obtained from the mixing of 100 µl 10^5 cells/ml (positive or negative for CDw52) with either 100 µl or 50 µl 20 µg/ml intact or deglycosylated (degly) NSO IgG1 and IgG4 in the presence or absence of anti-human IgG (aHuIg) 20 µg/ml.

	Thymidine incorporation (% control)	
Antibody used	**Jurkat cells**	**Jurkat + CDw52 cells**
NSO.IgG1	94.3%	79.2%
NSO.IgG4	93.5%	79.6%
Deglyco NSO.IgG1	90.8%	88.2%
Deglyco NSO.IgG4	95.9%	90.2%
anti-HuIg	96.9%	96.5%
NSO.IgG1+a-HuIg	98.9%	50.3%
NSO.IgG4+a-HuIg	97.9%	55.1%
Degly NSO.IgG1+aHuIg	98.9%	51.8%
Degly NSO.IgG4+aHuIg	100.4%	64.4%
control	100%	100%

From Table 4.6, it can be seen that intact NSO.IgG1 and IgG4 exhibited 79% control value incorporation (21% inhibition) when administered un-crosslinked to transfected cells. The incorporation of the transfected cells decreased to 50% and 55% of control value (50 and 45% inhibition) respectively, in the presence of anti-human IgG. The deglycosylation of IgG1 antibody did not substantially change the response whereas the deglycosylation of IgG4 reduced the inhibition seen with intact crosslinked antibody.

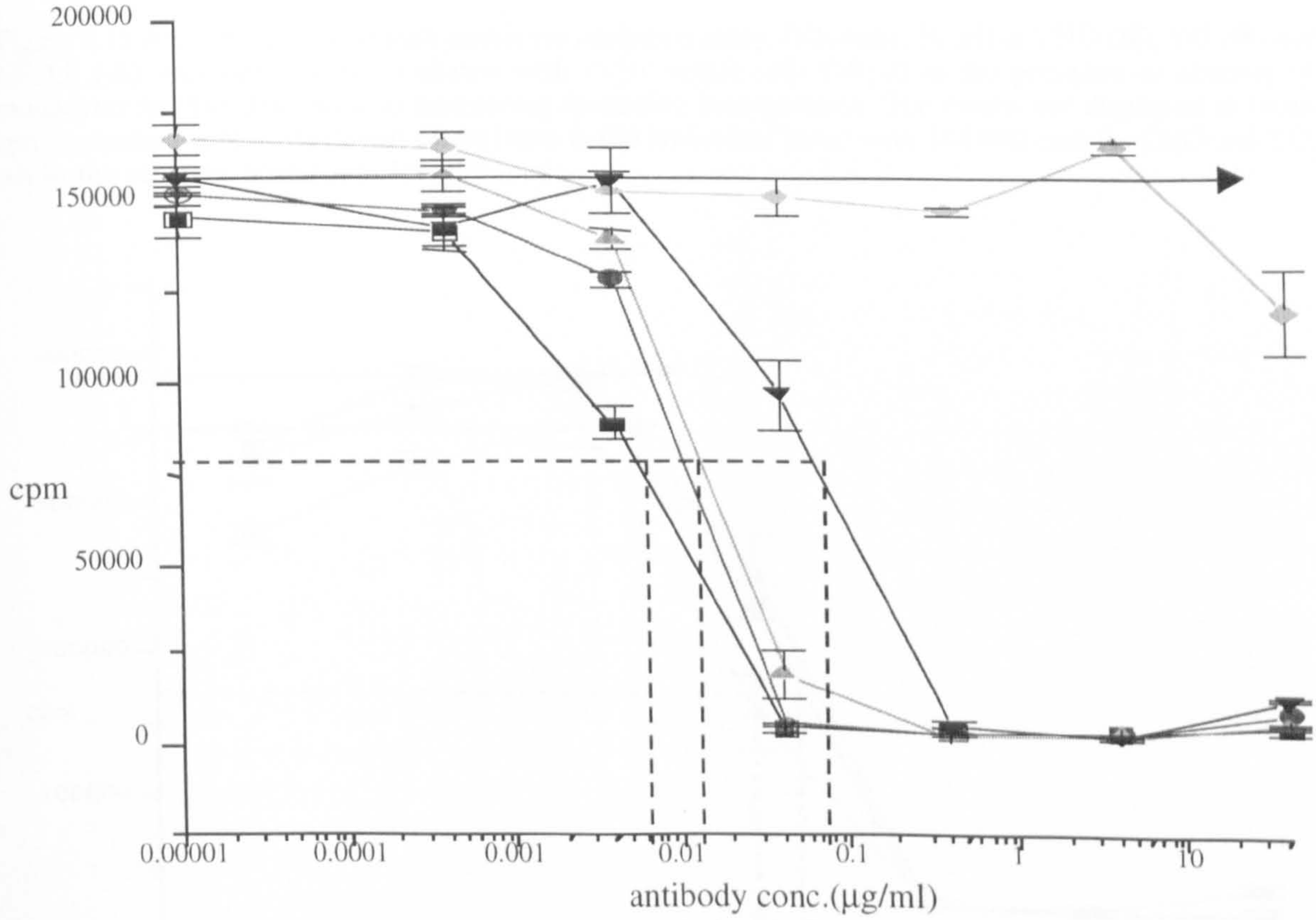
4.4.4 Monocyte cytostasis assays

As before the antibodies were placed into assays in comparison with CHO Campath as and when produced and then later into single comparative assays.

4.4.4.1 Comparison of intact and deglycosylated NSO.PF Campath-1H with CHO-derived antibody

Assays, utilising monocytes isolated 3 weeks previously, compared intact and deglycosylated NSO.PF#2 Campath-1H with CHO-derived antibody. YO antibody was included within the experiment as was anti-HLA antibody. The results of a typical assay are displayed in Fig.4.14.

Figure 4.14 A comparison of NSO.PF with YO and CHO Campath-1H for induction of monocyte cytostasis. Monocytes cultures or medium were incubated with 50 µl $8x10^4$ Wien 133 cells/ml in the presence of either 50 µl dilutions of intact CHO (■), YO (●) NSO.PF (▲) Campath or a-HLA (◆) antibody or deglycosylated NSO.PF (▼). Thymidine incorporation was monitored after five days. The results illustrated are in the form of mean cpm ± standard errors. Maximum thymidine incorporation (solid horizontal line) was taken as 160,000 cpm.



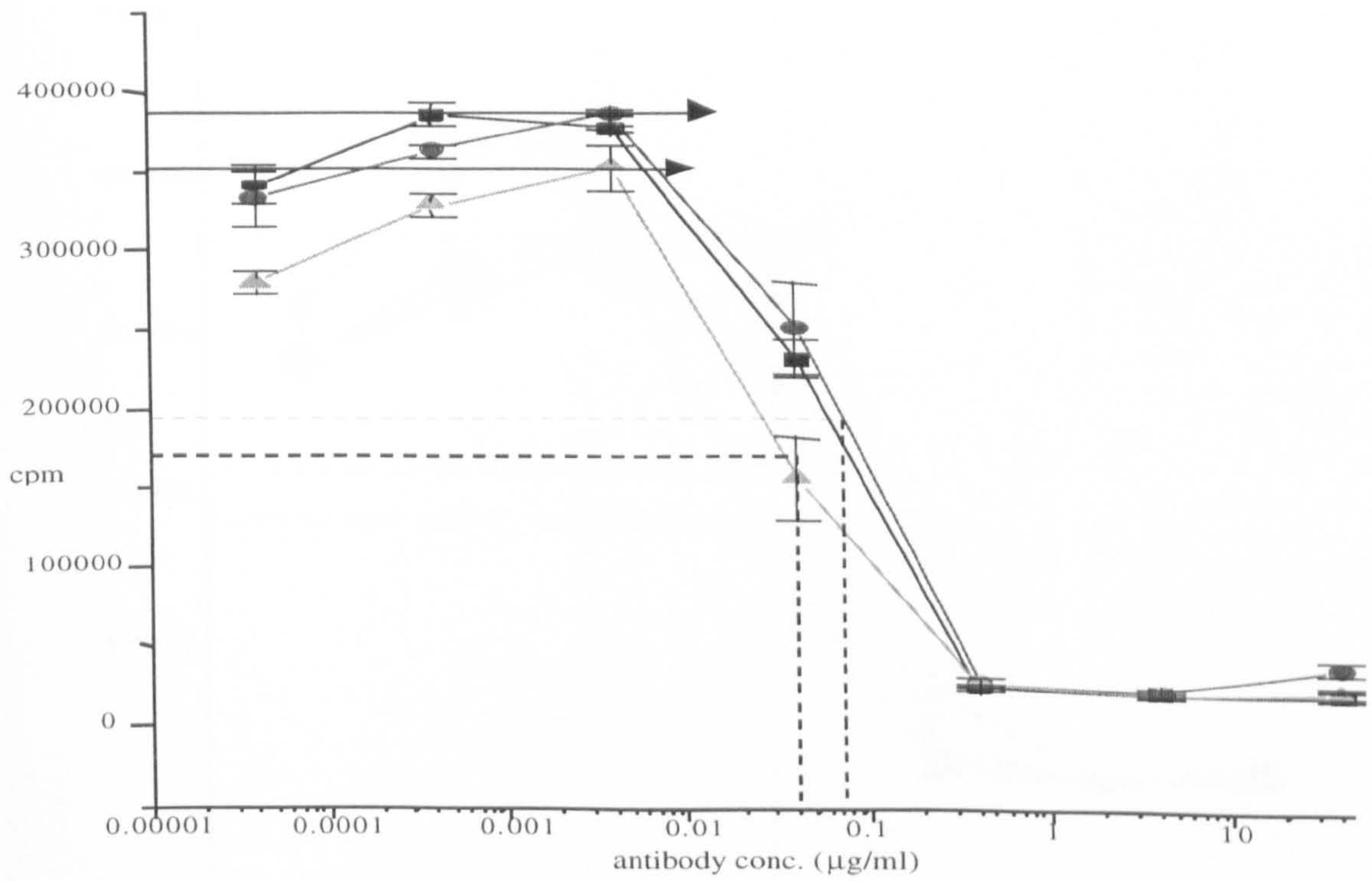
50% inhibition was generated at concentrations of 0.006 µg/ml for CHO, 0.015 µg/ml for YO and NSO.PF whilst 0.08 µg/ml of deglycosylated NSO.PF was required.

Comparing the results with those described for CHO Campath-1H in Chapter three (section 3.6.1), all of the intact antibodies perform as expected in the assay. Monocytes inhibited the target cell thymidine uptake to varying degrees depending on Campath concentration. CHO, YO and NSO.PF antibodies mediated maximum inhibition at greater than 0.04 µg/ml. The concentrations of intact antibody required to achieve 50% inhibition were as follows; CHO 0.006 µg/ml, YO and NSO.PF 0.015 µg/ml. The deglycosylation of NSO.PF reduced but did not ablate its ability to induce cytostasis as demonstrated by a shift in the concentration response curve. In terms of antibody required to achieve 50% inhibition the amount increased from 0.015 mg/ml (intact) to 0.08 µg/ml for deglycosylated. The fact that the antibody was able to induce inhibition is in sharp contrast with the failure of deglycosylated antibody to induce ADCC responses.

4.4.4.2 Comparisons of CHO, YO and NSO.F Campath in monocyte cytostasis assays

In experiments similar to those in 4.4.4.1, CHO and YO Campath-1H was compared with NSO.F in assays utilising monocytes which had been in culture for two weeks. The results displayed in Fig.4.15 are the mean of triplicates (cpm) plus or minus standard errors.

Figure 4.15 A comparative Campath monocyte inhibition assay. Dilutions (50 µl) of CHO (■), YO (●) and NSO.F (▲) antibodies were incubated with $4x10^3$ target cells (50 µl) in the presence or absence of monocytes for five days prior to monitoring thymidine incorporation. The results are displayed as mean cpm ± standard errors. Maximum control cpm (solid horizontal lines) were 380,000 cpm for CHO and YO, whilst the value for NSO.F was 350,000 cpm.



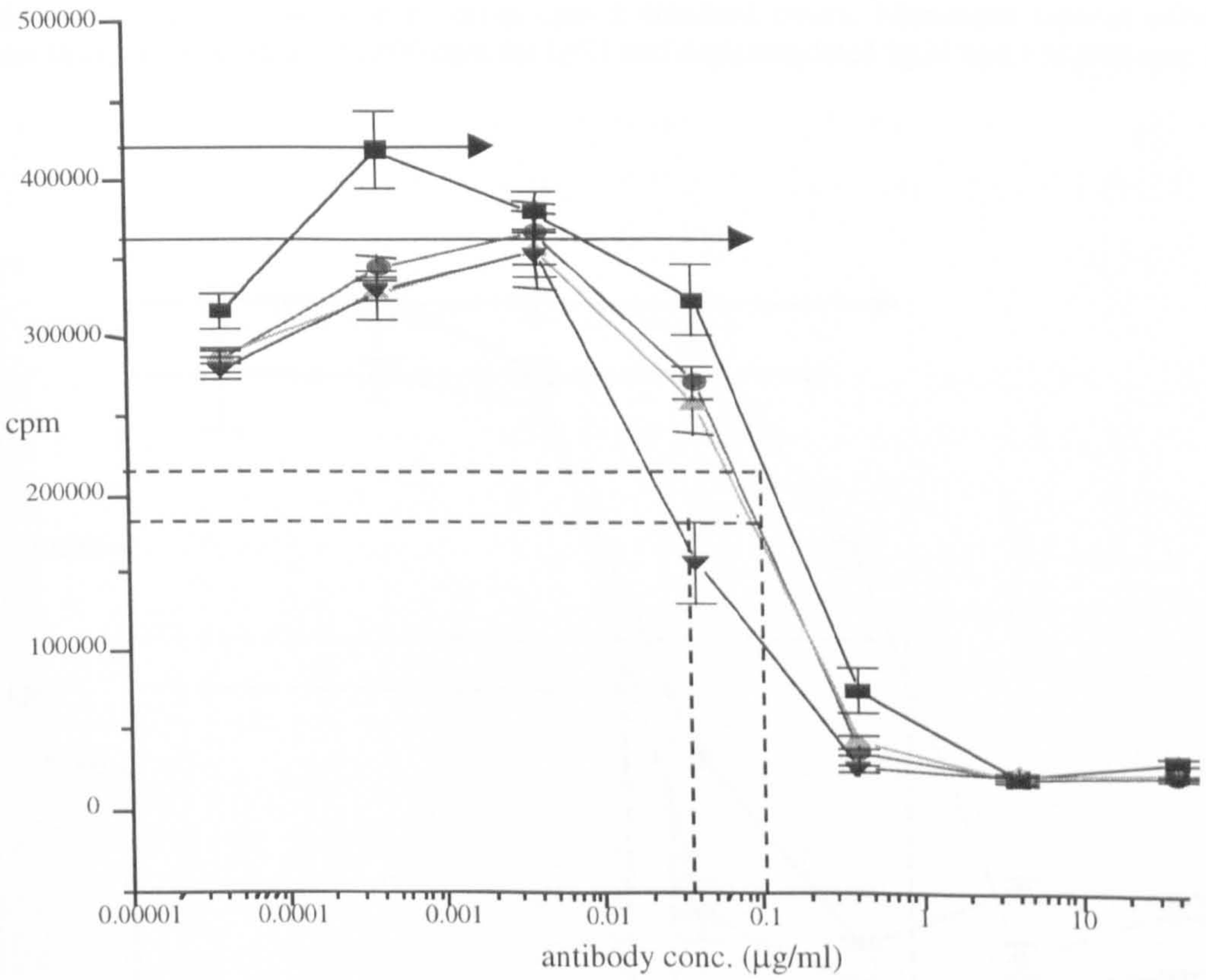
50% inhibition was generated with 0.07 µg/ml CHO or YO Campath and 0.04 µg/ml NSO.F.

In Fig.4.15, all of the antibodies tested induced maximum monocyte inhibition of Wien 133 thymidine incorporation at greater than 0.3 mg/ml. A value of 50% inhibition (175000 cpm) was achieved with 0.07 mg/ml CHO or YO antibody. The NSO.F Campath only required 0.04 mg/ml to generate the same 50% value. In the absence of monocytes (data not shown), concentrations of CHO, YO and NSO.F antibody did not depress the thymidine incorporation of the target cells.

4.4.4.3 NSO Campath isolates compared in monocyte cytostasis assays

NSO.S#1, NSO.PF#2, NSO.CF and NSO.F Campath-1H were compared to detect changes in antibody action when isolated from cells cultured under different conditions and in different media. The results are illustrated in Fig.4.16 and were derived from the same experiment as Fig.4.15.

Figure 4.16 A monocyte cytostasis assay comparing Campath NSO.S (■), NSO.PF (●), NSO.CF (▲) and NSO.F (▼). Dilutions (50 µl) of the antibodies were mixed with $4x10^3$ Wien 133 cells in the presence or absence of monocytes and incubated for five days prior to monitoring thymidine incorporation. The results are illustrated as mean cpm ± standard errors. Maximum control values (solid horizontal lines) were 420,000 cpm for NSO.S and 360,000 cpm for NSO.PF, CF and F.



50% inhibition was achieved with 0.035 µg/ml NSO.F whilst 0.1 µg/ml of the remaining antibodies was required.

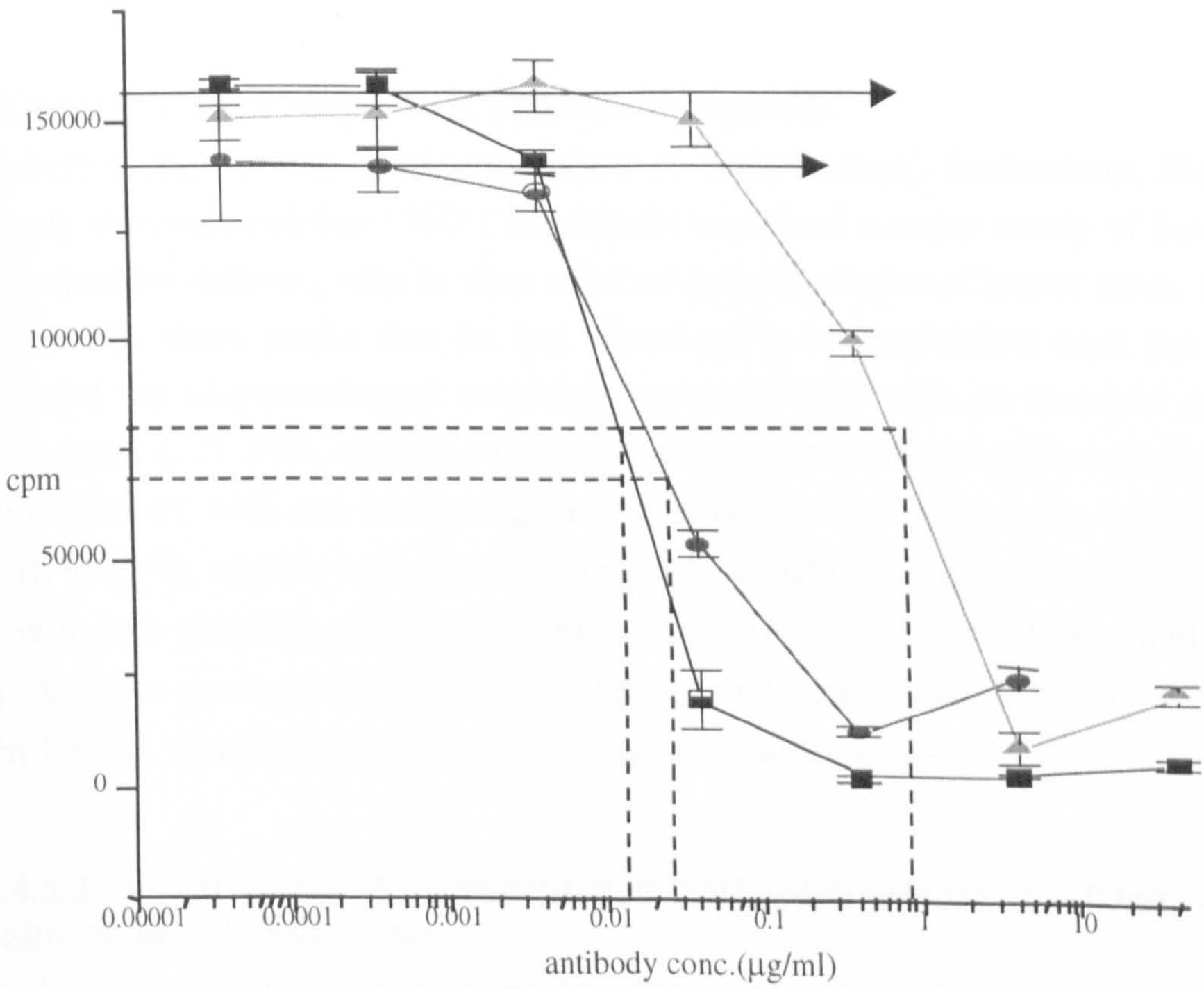
Comparisons of the different NSO antibodies in the same monocyte cytostasis experiment indicates that there are minor differences in their ability to induce cytostasis which may be a consequence of their modes of culture. NSO.PF, .CF and .S are the least effective antibodies in this assay, requiring 0.1 µg/ml to achieve 50% inhibition. The most active isolate is the antibody isolated from cells cultured under fermentor conditions

i.e. NSO.F Campath, which induces 50% inhibition at 0.035 μg/ml. In the absence of monocytes, none of the antibodies exhibit toxicity at high concentrations (data not shown).

4.4.4.4 Intact NSO Campath IgG1 compared with intact or deglycosylated IgG4

Intact NSO.PF#1 IgG1, NSO.IgG4, plus deglycosylated IgG4 antibodies were compared in monocyte assays. IgG4 was found to be active in inducing cytostasis in the presence of monocytes (Fig.4.17).

Figure 4.17 IgG1 and IgG4 compared in a monocyte cytostasis experiment. Dilutions (50 μl) of either intact IgG1 (■), IgG4 (●) or deglycosylated IgG4 (▲) were incubated with $4x10^3$ Wien 133 cells in the presence or absence of monocytes for five days prior to monitoring thymidine incorporation. The results are with monocytes and expressed as mean cpm ± standard errors. Maximum control values (solid horizontal lines) were taken as 160,000 cpm for IgG1 and deglycosylated IgG4 and 130,000 cpm for intact IgG4.



Concentrations of antibody generating 50% inhibition were 0.85 μg/ml deglycosylated IgG4, 0.015 μg/ml IgG1 and 0.025 μg/ml IgG4.

The concentrations of antibody necessary for 50% inhibition of the target cell incorporation were 0.015 μg/ml for IgG1 and 0.025 μg/ml for IgG4. Deglycosylation of the IgG4 Campath-1H depressed, but did not completely ablate, the response as was also

found for NSO.PF IgG1 antibody. To achieve 50% inhibition, 0.85 µg/ml of deglycosylated IgG4 antibody was required, representing nearly a ten fold increase over the 0.08 µg/ml of deglycosylated NSO.PF IgG1 required (see section 4.4.4.1).

4.4.5 Carbohydrate comparisons

During the media development for NSO Campath-1H, the majority of each antibody sample was compared, with both YO and CHO Campath-1H, for biological function in a series of experimental assays. In parallel to these assays, small amounts of each antibody were also prepared for carbohydrate analysis. The antibodies were freeze-dried and the N-linked CH2 domain carbohydrate molecules cleaved from the peptide by the method of hydranzinolysis. The resulting oligosaccharides were isolated from the peptide and to reduce the complexity of interpretation, treated with trifluoracetic acid to remove terminal sialic acid residues. The oligosaccharides were then tested for carbohydrate composition by both LDMS, to determine molecular weight, and Dionex HPAEC.

4.4.5.1 The CHO Campath-IH carbohydrate profile

Dr. Robert Lifely, of the Wellcome Research Laboratories, Beckenham, Kent, had previously demonstrated that CHO Campath-1H contained a major family of fucosylated oligosaccharides differing only in their terminal galactosylation (Chapter three, Lifely et al 1995). The three peaks that he had observed to be associated with the peptide represented the oligosaccharide structures either 004301 with no terminal galactose residues (peak I, 21.39%, relative retention time 0.59) and of molecular weight 1486.6, structure 014301 with one terminal galactose residue and of molecular weight 1648.8 (peak II, 40.29%, relative retention time 0.67) and structure 024301 of molecular weight 1811 with two terminal galactose residues (peak III, 15.42%, relative retention time 0.78). A minor monogalactosylated non-fucosylated component, 014300 of molecular weight 1502.6, was also seen in the CHO oligosaccharide sample.

4.4.5.2 The carbohydrate composition of NSO antibodies isolated from cells cultured in different media

When investigating the carbohydrate associated with the antibody isolated from the antibody transfected NSO cells, it was observed that as the cells were adapted to different culture mediums, the N-linked carbohydrate moieties altered (HPAEC, Fig.4.18 and LDMS, Fig.4.19). The samples tested were NSO.S#1, NSO.PF#2 and NSO.CF.

Figure 4.18 The HPAEC profiles of carbohydrate isolated from different NSO antibodies. The N-linked oligosaccharides of a)NSO.S, b) NSO.PF or c) NSO.CF were analysed by HPAEC in relation to a known standard i.e. neuraminic acid (NeuNAc). The standard is indicated by the peak at either 13.283 (S), 13.408 (PF) or 13.35 (CF) minutes on each profile. The highlighted peaks I, II and III, refer to the three forms of oligosaccharide found on IgG.

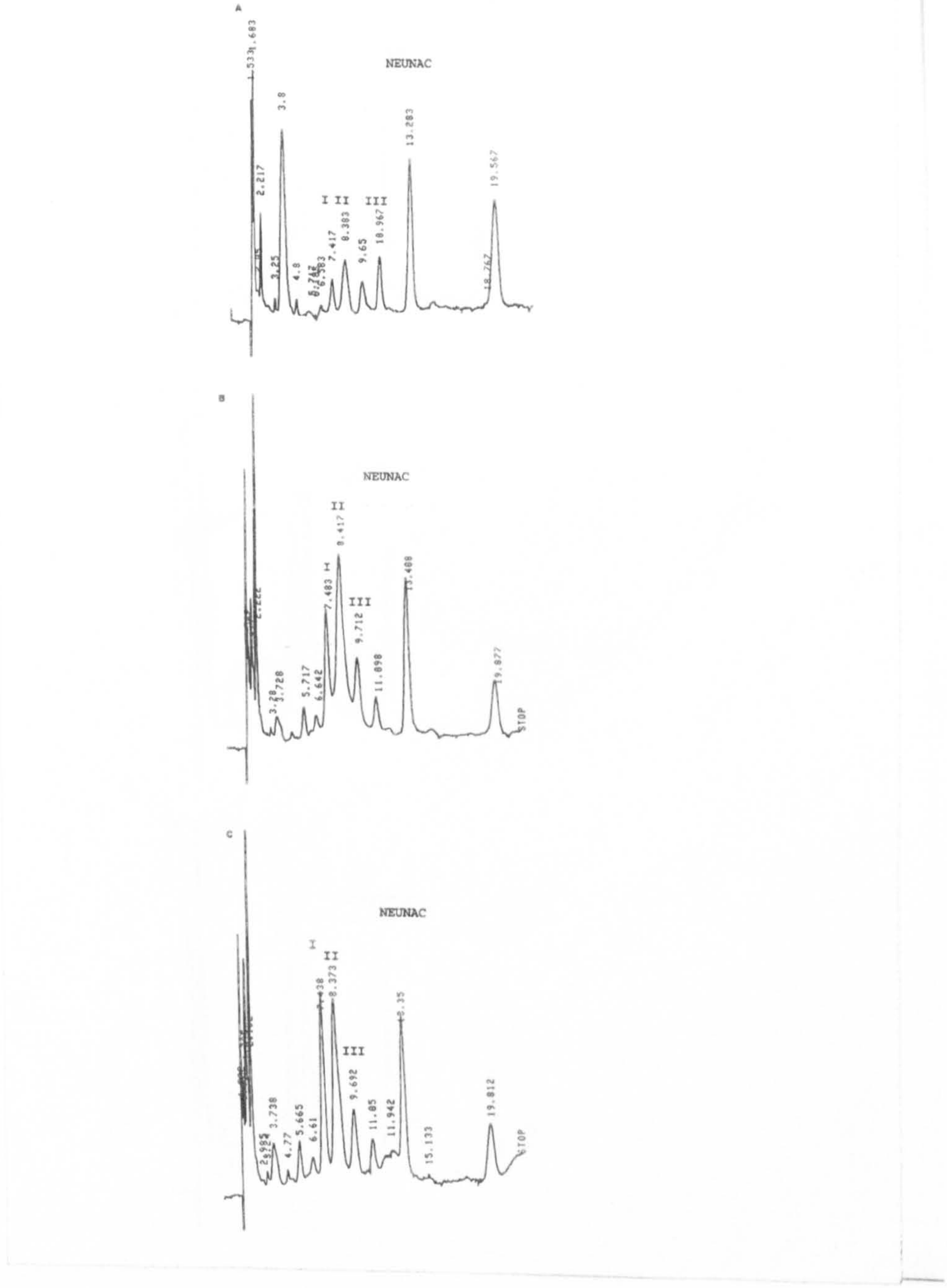
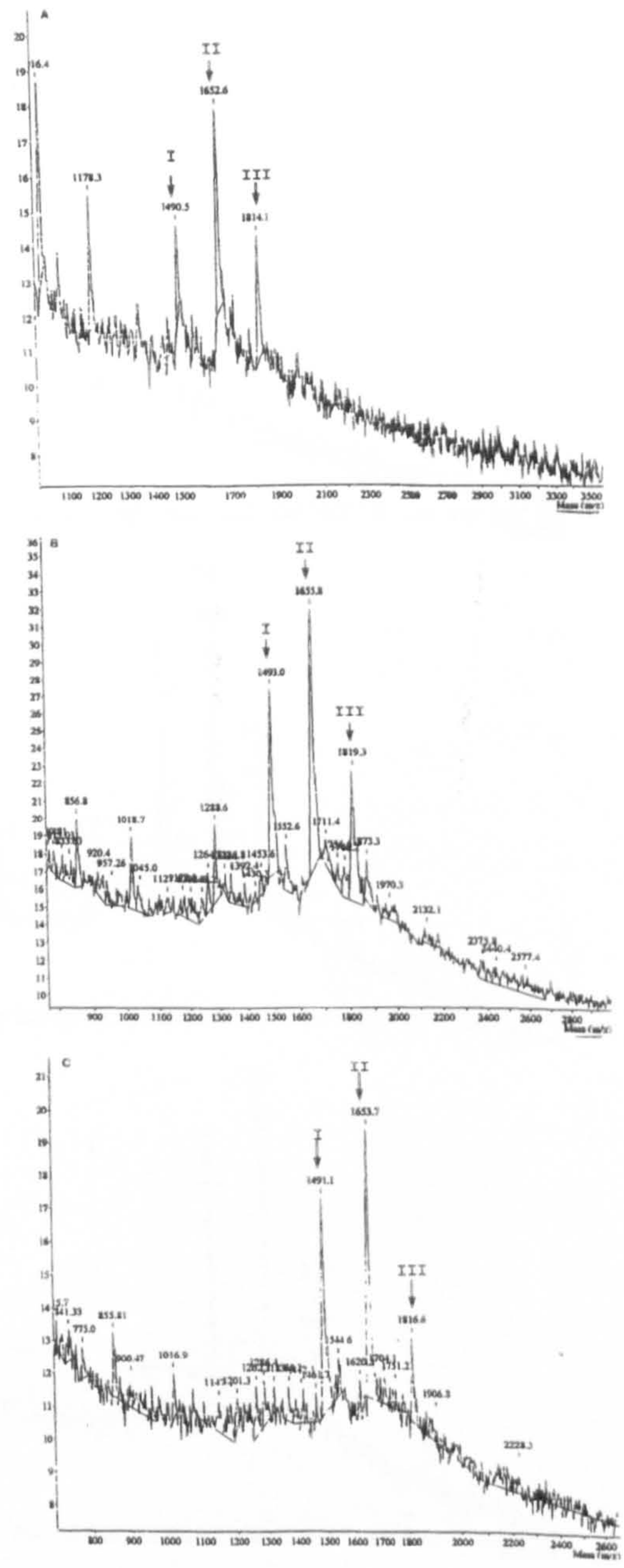


Figure 4.19 The LDMS profiles of the carbohydrate from different isolates of NSO antibodies. The N-linked oligosaccharides of a) NSO.S, b) NSO.PF) or c) NSO.CF were analysed by LDMS. The highlighted peaks I, II and III, refer to the three forms of oligosaccharide found on IgG.



By HPAEC analysis (Fig.4.18), the three oligosaccharide peaks observed in all NSO antibodies were at the time points 7.417 (S), 7.483 (PF) and 7.438 (CF) minutes for peak I, 8.383 (S), 8.417 (PF) and 8.373 (CF) minutes for peak II or 9.65 (S), 9.712 (PF) and 9.692 (CF) minutes for peak III. By division of the experimental sample retention time with that of the reference it was possible to devise a relative retention time value for each profile and thus extrapolate the structure/molecular weight by comparison to defined oligosaccharide standards. When these were calculated, the relative retention times for

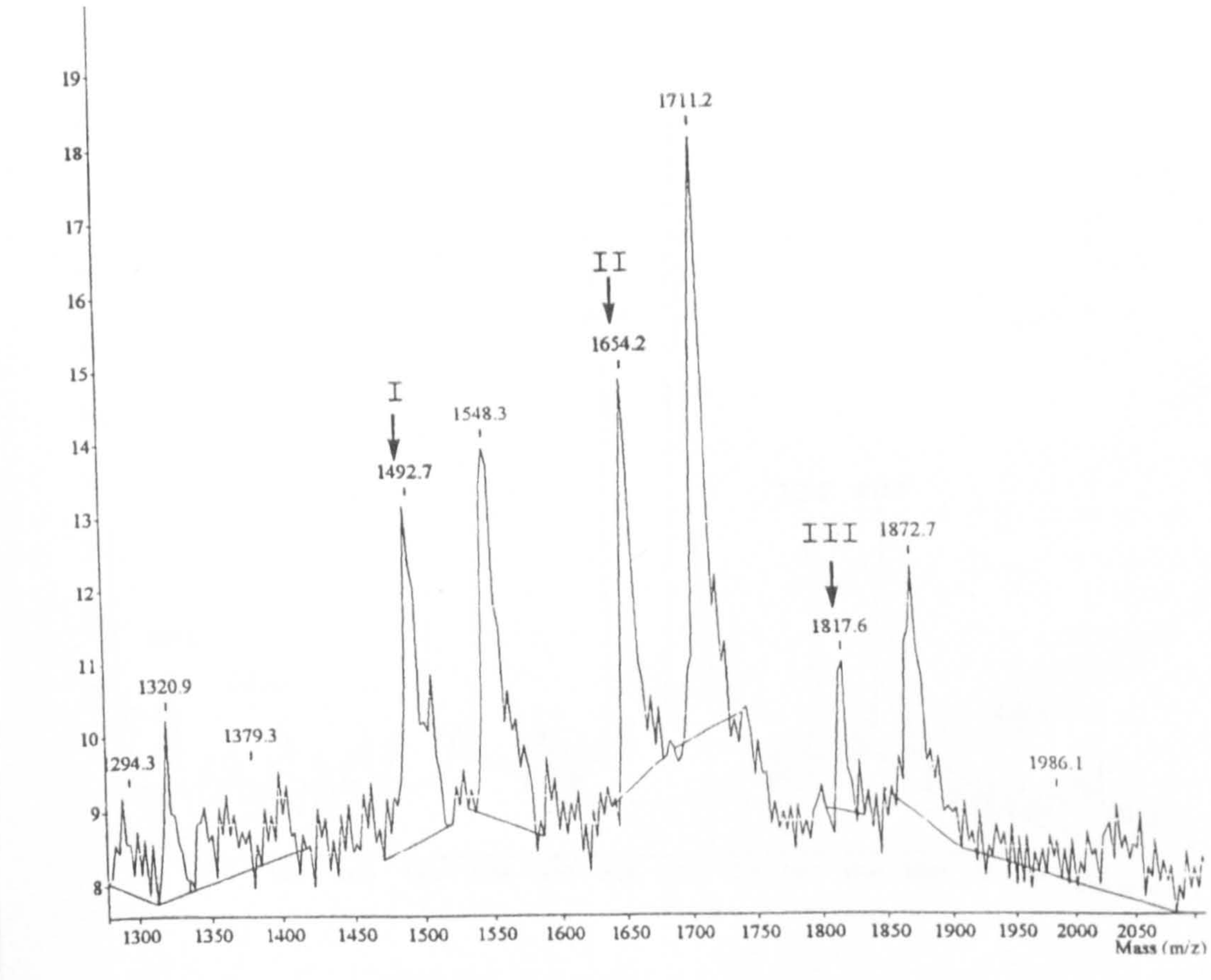
the three peaks were 0.56 for peak I, 0.63 for peak II and 0.73 for peak III. These were all within the same range as the peaks seen for CHO Campath-1H and suggested that the same oligosaccharides are present. When aliquots of the same samples were analysed by LDMS (Fig.4.19), peaks at the molecular weights of 1490.5 (S), 1493 (PF) and 1491.1 (C) for peak I, 1652.6 (S), 1655.8 (PF) and 1653.7 (CF) for peak II and finally 1814.1 (S), 1819.3 (PF) and 1816.6 (CF) for peak III were identified which were also close to the molecular weights of the peaks observed within the CHO material (1486, 1649 and 1811).

When the overall HPAEC and LDMS profiles for each sample were compared it was observed that the height and width of the individual peaks varied in relation to each other, reflecting changed composition ratios. In NSO.S (from cells cultured in serum), the most prominent peak was peak II (the mono-galactose), whilst peaks I (agalactosyl) was minimal and peak III (di-galactose) intermediate. This profile altered for NSO.PF (cells cultured in protein-free medium) in that peak II remained the most prominent but, peak I became more abundant than III. Further changes occurred when the cells were transferred to cholesterol-free medium, such that for NSO.CF a minor level of peak III was observed with prominence of both peaks I and II, with peak II slightly more abundant. Thus, as the content of peak I increased and peak lll decreased, the antibodies appeared to become more active in ADCC. The observed percentages of the different peaks are summarised in Table 4.7.

4.4.5.3 The carbohydrate content of the fermentor batch of NSO IgG1

Analysis of NSO.F revealed that the peaks identified by LDMS in the earlier NSO antibodies were shadowed by intermediate peaks of 1548.3, 1711.2 and 1872.7 (Fig.4.20). These represent structures within the possible range for the oligosaccharide structures 004310, 014310 and 024310 i.e. non-fucosylated bisecting oligosaccharides with no, one or two galactose residues. The ratios of the major fucosylated peaks were observed to be: peak II (mono-galactose) the most abundant at 28.45%, peak I (agalactosyl) of intermediate amount 16.69% and peak III (di-galactose) the minor component 10.56% and these are summarised in Table 4.7.

Figure 4.20 The LDMS profile of the carbohydrate isolated from NSO.F. The N-linked oligosaccharide isolated from antibody prepared from fermentor grown cells was analysed by LDMS. The highlighted peaks I, II and III refer to oligosaccharides found on IgG.



Dr. Lifely later re-analysed the NSO.F material (Lifely et al 1995) by Dionex HPAEC and also found the same families of oligosaccharides to that seen in the CHO antibody and confirmed that they differed in ratio. The ratios observed were similar to those seen for NSO.PF and further analysis of the intermediate peaks, after single or combination exoglycosidase treatments to reduce the oligosaccharides to more simple structures (data not shown) demonstrated them to be products of insufficient cleavage during hydranzinolysis and not bisecting oligosaccharides. However, a minor fraction of under glycosylated oligosaccharides which migrated with shorter retention times was detected.

4.4.5.4 YO Campath-1H

In identical experiments to those mention previously, Dr. Lifely investigated the structure of Campath-1H isolated from YO cells. The YO antibody had a more complex profile than either the CHO or NSO material (data not shown, Lifely et al 1995). The peaks isolated from the YO antibody represented not only the major fucosylated peaks seen in both the CHO and NSO samples in the ratio 6.4% agalactosyl, 16.54% monogalactosyl

and 7.56% digalactosyl oligosaccharides but also additional non-fucosylated and fucosylated bisecting glucosamine (GlcNAc) series (004310, 004311, 014310, 014311, 024310 and 024311) as determined by exoglycosidase treatments.. Human IgG1 is known to contain approximately 18% bisecting glucosamine residues (Furakawa et al 1991). The percentages of the three major YO peaks are tabulated in Table 4.7.

Table 4.7 A summary of the relative percentages of isolated oligosaccharide peaks present in carbohydrates released from different Campath-1H antibodies. Each peak is presented as percentage (%) of total oligosaccharide plus as a % of the three peaks

	peak I agalactosyl		peak II monogalactosyl		peak III digalactosyl	
Antibody used	%	as % of (I+II+III)	%	as % of (I+II+III)	%	as % of (I+II+III)
CHO	21.39%	28	40.29%	52.3	15.42%	20
YO	6.4%	21	16.54%	54	7.56%	24
NSO.S	< 1%	9.8	6.38%	62	2.87%	28
NSO.PF	8.38%	22	22.64%	60	6.59%	17.5
NSO.CF	8.17%	34	12.21%	50.8	3.64%	15.2
NSO.F	16.69%	29	28.45%	51	10.56%	18.9

4.5 Discussion

The cloning of the humanised Campath heavy and light antibody fragments into the Celltech vectors was not straight forward. The initial phase of transferring the antibody inserts from their pUC based holding vectors was problem free, but later manipulations were more difficult. The guidelines supplied with the vectors suggested that, when the vector pEE6hcmv-*Bgl II* containing the correct orientation of heavy chain was obtained, it should be cleaved with the enzymes *Bam HI* and *Bgl II* and the released fragment re-inserted into the *Bam HI* digested vector pEE12 containing the correctly orientated light chain. This would result in a plasmid in which both heavy and light chain sequences were expressed by their own control elements, in the same direction, with no intervening sequences. However, the recommended step of cloning the *Bam HI / Bgl II* digested fragment from pEE6 into *Bam HI* digested pEE12 plus light chain proved impossible to achieve. After numerous attempts, an alternative solution was devised in which the vector containing the heavy chain was digested with the enzymes *Bgl II* plus *Sal I* and the released fragment ligated into the light chain bearing pEE12 digested with *Bam HI* and *Sal I* . This resulted in a similar plasmid, pEE6H12L, in which both antibody fragments were expressed by their own control sequences in the same direction but with two hundred base pairs of intervening sequence between them.

The expression of pEE6H12L was also not without problems. It was unexpected that so many of the colonies resulting from the electroporation of the plasmid into NSO cells would result in non producing lines, even under selection. Unfortunately it took several weeks to discover this in each case. An additional problem associated with the expression and purification of the resulting antibodies was the culture medium. When purifying the antibody from serum containing medium, on a laboratory scale utilising FPLC with either protein A or G columns, contaminating bovine IgG became a problem for both the isolation and the spectrophotometric quantitation. The latter situation was overcome by the derivation of the anti-human IgG ELISA for quantitation but the former problem was more complex and required the development and manufacture of a specific protein-free medium (Keen and Hale 1996). The development of the medium did however provide the ideal conditions for the close study of the evolution of the antibody properties in terms of both carbohydrate composition and associated biological activity.

From the initial studies on CHO antibody it had become obvious that it would be difficult to define the activity of an antibody in reliable terms in a single assay. Each aliquot of effector cells appeared to have an inherent variability when those cells were placed in standard assays. However when compared with both CHO and YO isolates of Campath-

1H, the NSO antibodies were also found to vary within assays depending on the method of their culture. As each step of the media development progressed, it was therefore essential to adapt the antibody producing NSO clones to that medium, grow the cells in a large batch and isolate that stage of antibody for analysis in several assays, in comparison with either or both CHO and YO Campath-1H. To compare the results it was also found advantageous to express the data in terms of 50% of maximum specific release values for the ADCC assays, where possible, or 50% inhibition for the engagement and crosslinking of antigen or monocyte cytostasis experiments.

In comparative ADCC assays (Figs.4.7.and 4.9), the earliest NSO antibody purified from cells cultured in serum containing medium was less efficient than CHO antibody. The amounts of antibody required to establish 50% of maximum specific lysis of target cells was 0.006 µg/ml. When NSO.PF antibody was isolated and compared with the standards, it was inferior at the 50% of maximum specific release level in that 0.015 µg/ml was required instead of the 0.001 and 0.00035 µg/ml for the CHO and YO antibodies respectively. Even when cultured under controlled conditions in a fermentor, the NSO.F antibody failed to equal either of the two standard Campath-1H antibodies and required 0.0015 µg/ml to achieve 50% of maximum lysis in comparison with 0.001 µg/ml for the CHO antibody and 0.00035 µg/ml for the YO material. The underlying mechanism of action of the NSO antibodies, in ADCC assays, was found to be the same as that for the CHO antibody in that blockade of the IgG Fc RIII on the mononuclear effector cells decreased the activity of the NSO.S and PF antibodies to a similar level as that of the CHO antibody in the concentration range 0.0002 to 0.2 µg/ml. As with the CHO antibody, the anti-Fc R antibodies used for blocking were not tested prior to the experiments and were therefore not shown to influence receptor function. Removal of the carbohydrate from the NSO antibody by N-glycanase treatment, totally ablated the ADCC response. As the IgG Fc RIII on the effector cell has been suggested to be the major receptor involved in ADCC action, it is possible that the antibody recognition or binding efficiency of that receptor is altered by glycosylation changes induced by culture conditions.

When comparing the ability of the different NSO isolates to induce effector cell-independent cytostasis of transfected Jurkat cells, there appears to be little difference when compared to either CHO or YO Campath-1H. The carbohydrate moieties on the Fc portion of the antibody were shown to not be necessary for antigen recognition as demonstrated by the substitution of the intact NSO.PF antibody with deglycosylated material. In the presence of the anti-human IgG crosslinking reagent, deglycosylated antibodies generated identical responses to those of the untreated antibody. Equivalent

antigen binding was confirmed by the application of dilutions of the different antibodies to CDw52 positive Wien 133 cells and detection with FITC-labelled mouse anti-human antibody.

Surprisingly, comparisons of the NSO isolates with CHO and YO Campath antibodies in monocyte cytostasis experiments demonstrated that the later stage NSO antibodies were sometimes more effective at inducing cytostasis than either of the CHO or YO material (Figs 4.15 and 4.16). This is different from the ADCC results. In these experiments NSO.F required 0.04 μg/ml, to achieve 50% inhibition of thymidine uptake, compared to 0.07 μg/ml for the CHO and YO antibodies. In the same assay, but different graph, the various NSO forms were checked against each other. NSO.F antibody (0.035 μg/ml) was necessary to achieve 50% inhibition whilst NSO.PF, CF and NSO.S antibodies needed 0.1 μg/ml. As seen with the ADCC results, the underlying mechanism of action remained the same as the CHO antibody, but unfortunately it was not possible to demonstrated receptor usage reliably. Deglycosylation of the NSO antibody reduced but, unlike ADCC, did not ablate, the response as was also demonstrated for the CHO antibody.

The carbohydrate analysis of the NSO antibody isolates revealed interesting data which could possibly explain the differences in biological properties. The first NSO antibody isolated, that from serum containing medium, was found to contain less than 1% agalactosyl, 6.38% monogalactosyl and 2.87% digalactosyl oligosaccharide. In ADCC assays this was the least active antibody. When the antibody was isolated from protein-free medium, both the activity and the carbohydrate profile altered. The NSO.PF antibody was demonstrated to perform more efficiently than NSO.S in ADCC assays, but remained less active than both CHO and YO Campath-1H. There was no improvement over the NSO.S antibody in the monocyte cytostasis experiments. At the carbohydrate level, the NSO.PF antibody was found to possess 8.38%, 22.64%, 6.59% (agalactostyl, monogalactosyl and digalactosyl oligosaccharides respectively). Cholesterol-free medium resulted in NSO antibody which was of similar activity to NSO.PF antibody in ADCC and monocyte assays whilst the carbohydrate composition varied slightly with 8.17% agalactosyl, 12.21% monogalactosyl and 3.64% digalactosyl oligosaccharides. The most active NSO antibody was that cultured under protein-free fermentor conditions. This antibody, NSO.F, contained 16.69% agalactosylated oligosaccharide, 28.45% monogalactosyl and 10.56% di-galactosyl. Whilst it was compatible with NSO.PF and CF in ADCC, it was superior in monocyte assays. When compared to CHO and YO antibodies, the NSO.F Campath was less active than both CHO and YO in ADCC but superior in monocyte experiments. Dr. Robert Lifely had previously demonstrated CHO and YO antibodies to possess agalactosyl, monogalactosyl and digalactosyl

oligosaccharide ratios of 21.39%, 40.29%, 15.42% (CHO) and 6.43%, 16.54%, 7.56% (YO) respectively (Lifely et al 1995). I suggest that as NSO antibody glycosylation becomes more similar, in ratio, to CHO its action in ADCC improves (see Table 5.7). YO antibody is very different from both CHO and NSO in terms of glycosylation in that it contains bisecting GLcNAc moieties.

It has been suggested that for ADCC activity, IgG Fc RIII on the effector cells is important, whilst all three IgG Fc receptors on the monocyte effector cells are indicated to interact to mediate cytostasis. Perhaps these results indicate differences in affinity. If the carbohydrate on the antibody is not of the correct structure, the overall antibody conformation might alter and affect the interaction with one receptor more critically than that of three. This may explain why NSO antibody is less active than CHO and YO Campath in ADCC, but more comparable in monocyte induced inhibition.

The comparison of NSO IgG1 with NSO IgG4 Campath-1H also highlights the fine balance that carbohydrate imposes on antibodies. Both antibodies share antigen specificity and sequence homology and yet IgG1 interacted with CD56 positive natural killer cells to mediate ADCC and IgG4 did not. Both IgG1 and IgG4 antibodies were, however, able to mediate monocyte cytostasis. Even after deglycosylation, both antibodies were able to mediate cytostasis of a reduced nature suggesting that Fc CH2 carbohydrate may only effect recognition of Fc RIII. Thus, the inability of an antibody to mediate lysis in a short-term in vitro ADCC assay may not necessarily guarantee that the antibody will not deplete target cells in vivo. Indeed it has subsequently been shown that an IgG4 version of Campath-1H does deplete peripheral blood lymphocytes in patients (Isaacs et al 1996). There are several structural differences between the two subclasses, one being that IgG4 has a slightly shorter upper hinge region than IgG1 and as such IgG4 does not activate complement whilst IgG1 does. Other differences between the two are post-translational modifications such as carbohydrate addition.

CHAPTER FIVE: RESULTS- CLONING AND EXPRESSION OF CAMPATH ANTIGEN (CDw52) IN MAMMALIAN CELLS: INTERACTION BETWEEN CDw52 AND CAMPATH-1H

5.1 Introduction

The target for Campath-1H is CDw52, an antigen expressed on mature lymphocytes, monocytes and sperm (Hale et al 1983). The cDNA sequence of CDw52 encodes a 24 amino acid leader sequence with a further 37 amino acids forming the protein coding sequences of CDw52 (Xia et al 1991). Post-translational modification and cleavage generates the mature CDw52 antigen, which is composed of a 12 amino acid backbone anchored to the cell surface by a GPI-anchor. Several GPI-anchored molecules have been implicated in cell activation and signalling and this is the case for CDw52 (Rowan et al 1995). The protein backbone of the antigen is known to be modified by N-linked glycosylation on the asparagine residue at position three, with the added carbohydrate contributing to the total molecular weight of 20-28 kD. Campath-1H recognises an epitope formed by the last three amino acids of the mature protein plus components of the GPI-anchor (Xia et al 1993b). However, despite these data being available, the cellular function of the CDw52 antigen is still unknown. In order to facilitate studies on the function of CDw52 and to assist in the generation of assays to quantitate the effects of Campath-1H antibody, a source of defined transfected cells was considered desirable.

5.2 Aims

Cell lines expressing CDw52, such as the B cell lymphoma Wien 133 (Nacheva et al 1987) and the T cell line HUT 78, are suspension cell lines which express high levels of CDw52 antigen, together with a variety of other surface antigens consistent with their lineage. This chapter describes the isolation of cDNA encoding CDw52 antigen from Wien 133 cells, the confirmation of the CDw52 gene DNA sequence and the cloning of the CDw52 gene into an expression vector at a site suitable for regulated, high level expression in mammalian cells. The cell lines selected as host for CDw52 expression were the adherent line CHO dhfr- B11 and the well characterised suspension T cell line, Jurkat J6. Unexpectedly two forms of Campath cDNA were identified during PCR analysis, these differing by two amino acids in the latter half of the cDNA sequence (this observation had also been made by

the Waldman group but was documented as a possible PCR artefact (Xia et al 1991). The encrypted signals within this region are those which control the site of GPI-anchorage (Moran and Caras 1991a and b). Additional experiments described here attempt to investigate the properties of the second form of CDw52 by the transfection of the alternative cDNA into cells and comparing the biological properties of the two forms of CDw52. Chimeric CD4/CDw52 plasmids were also utilised to determine the possible role of the alternative GPI signal sequences.

Results

5.3 The isolation of cDNA encoding the CDw52 Antigen from Wien 133 cells

To isolate the cDNA for CDw52 from Wien 133 cells, two PCR primers based on the reported DNA sequence were synthesised (Fig.5.1). The forward primer (#3683) corresponded to the 5' sequences of the published coding strand and incorporated a *Hind III* site upstream from the ATG, the reverse primer (#3684) was complementary to the 3' sequences of the non-coding strand and incorporated an *Eco R1* site just downstream from the stop sequence. Utilisation of different restriction sites in the strategy allowed for the directional cloning of the resulting 206 bp. DNA fragment.

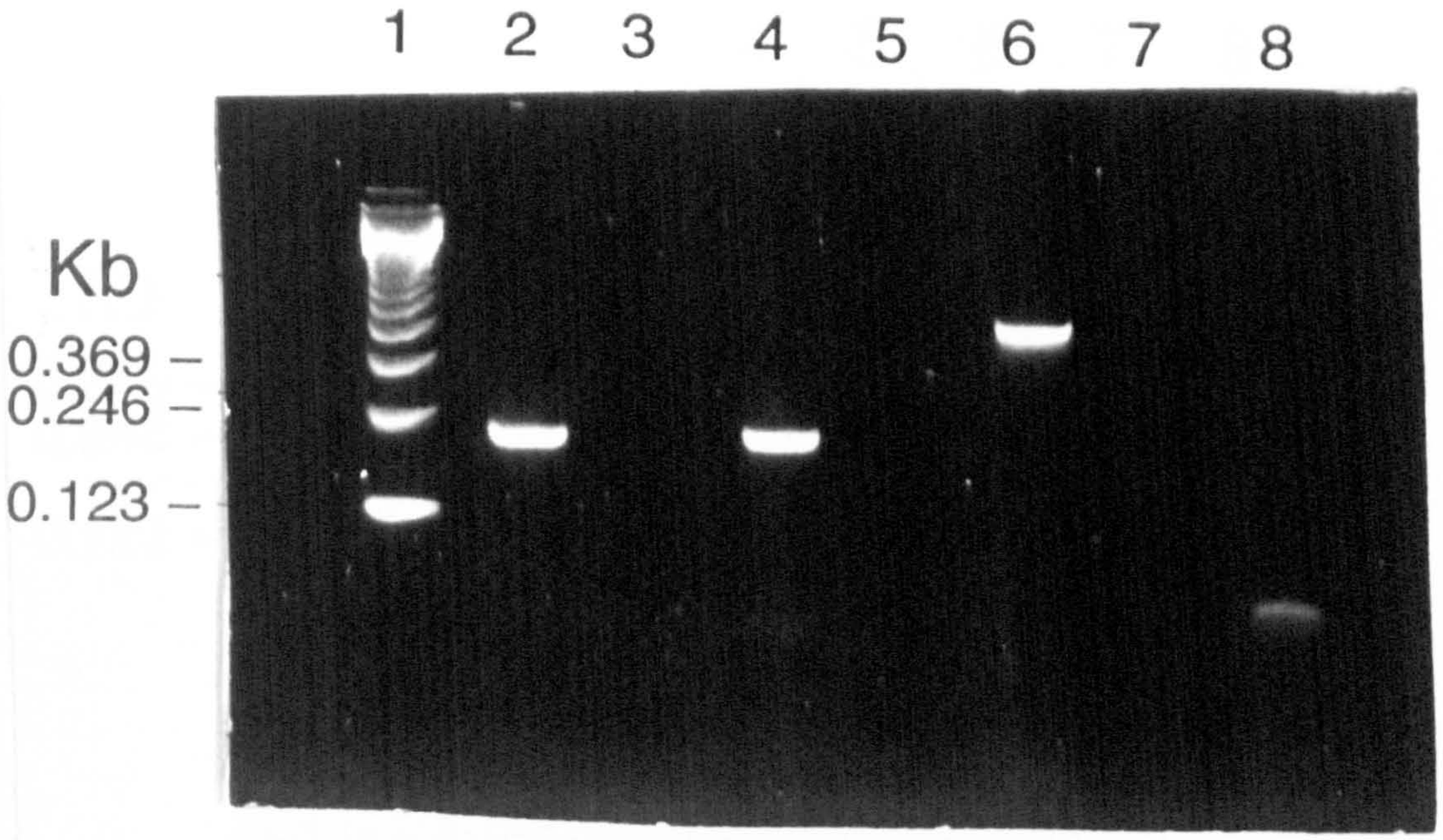
Figure 5.1 The PCR primers used for isolating the CDw52 antigen. The bases in bold type represent restriction sites.

The 31-mer 5' primer (#3683)	5' GATC**CAAGCTT**TCCTACCAAAATGAAGCGCT 3'
The 30-mer 3' primer (#3684)	5' GATC**GAATTC**TCAACTGAAGCAGAAGAGGT 3'

Total RNA was prepared from Wien 133 cells and mRNA was enriched using magnetic bead separation. A Gibco BRL 'SuperScript' kit was used to produce both the initial first strand cDNA from the resulting mRNA and then to synthesise a second strand DNA copy. A PCR performed on 100ng of the cDNA, utilising the primers described above, generated an expected single DNA fragment of 206 bp, which was purified on an agarose gel after

cleavage with *Hind III / Eco RI* (Fig.5.2). The purified DNA fragment was cloned into *Hind III / Eco RI* digested pUC18 for sequencing.

Figure 5.2 Agarose gel from which CDw52 cDNA was isolated. The lanes are as follows; Lane 1 123bp markers, lanes 2 and 4 the PCR product from 10µl Wien 133 cDNA using primers's #3683 and #3684, lanes 3 and 5 the PCR control using 10µl Wien 133 cDNA in the absence of primers, lane 6 the kit control PCR product of 1µl Lambda DNA using relevant primers, lane 7 the kit control using 1µl Lambda DNA in the absence of primers, lane 8 the PCR product from 1µl Wien 133 DNA using primers #3683 and #3684.



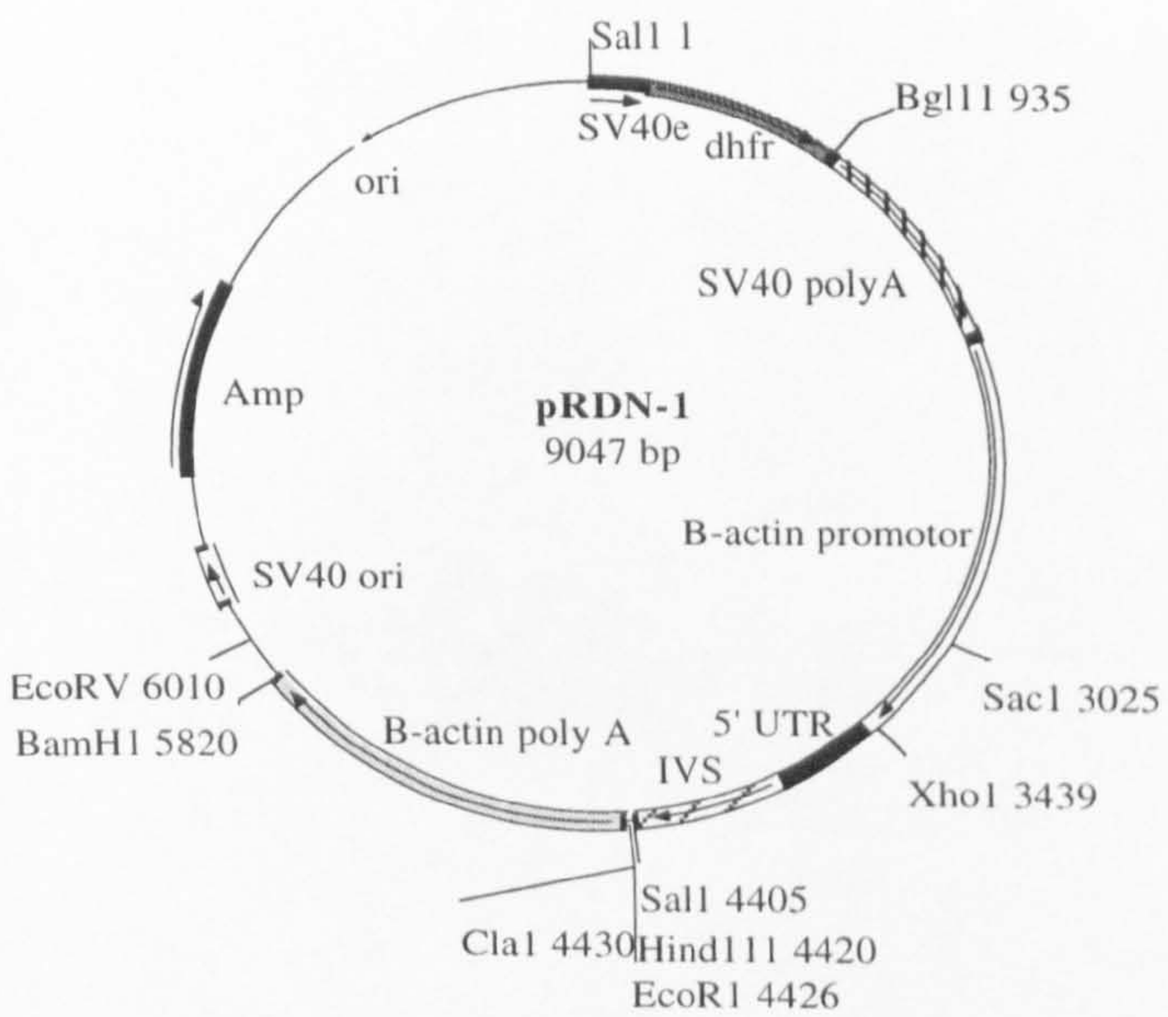
Sequencing of individual transformants from the cloning described above revealed that both the upstream and the coding region exactly matched the published DNA sequence of CDw52 except that at a position just four amino acids downstream from the point of GPI-anchor attachment (at amino acids numbered 16 and 17), forty-six percent of the independent DNA molecules sequenced had a DNA sequence change that resulted in a change in the encoded amino acids from asparagine and isoleucine to serine and methionine respectively. Subsequent studies used DNA which conformed to the published authentic sequence for CDw52 (Xia et al 1991).

5.4 Cloning and expression of the CDw52 cDNA in mammalian cells

Campath cDNA was excised from pUC18 and cloned into an expression vector pRDN-1 (Fig.5.3). pRDN-1 encodes the strong β-actin promotor and polyadenylation signals either

side of a polylinker encoding both *Hind III* and *Eco RI* sites. Thus, it was possible to insert the CDw52 open reading frame in the correct orientation. pRDN-1 also encodes a di-hydrofolate reductase (dhfr) gene cassette which permits the selection and amplification of antigen expressing colonies. After confirming the structure by restriction analysis a plasmid encoding CDw52 was named pRDN cAG#35 (later referred to as pRDN AG only).

Figure 5.3 A diagrammatic representation of the expression vector pRDN-1.3 The named restriction sites are those routinely used for orientation of inserts. *Hind III* and *Eco RI* were utilised for the directional cloning of the Campath antigen CDw52.



pRDNcAG#35 DNA was used to transfect several cell lines, initially alone into the CHO dhfr$^-$ line B11 and subsequently into the T cell line Jurkat J6 by cotransfection with a plasmid encoding neomycin resistance, p321 Neo.

5.4.1 Transfection into the CHO dhfr$^-$ B11 cell line

CHO dhfr$^-$ cells transfected with pRDN cAG#35 gave detectable surface expression of CDw52 antigen with a wide range of levels, as assessed by FACS analysis (Fig.5.4). The addition of increasing amounts of MX into the culture medium led to the selection of amplified expression in pooled positive cells. After dilution cloning, individual clones with high level CDw52 expression (Fig.5.5) were selected for further work. The 10^{-6}M MX

clone 10/D4, which had arisen from the 10 cells/well dilution cloning plate, was the most frequently used cell line in the following experiments.

Figure 5.4 A typical FACscan of CHO dhfr$^-$ cells transfected with the plasmid pRDN AG. The upper histogram is representative of untransfected cells. The bottom histogram demonstrates the specific expression of cells transfected with the cDNA of CDw52. 100 µl of 10^5 cells per ml were stained with either 10 µl of a 1 in 10 dilution of FITC-Campath-1H or PBS. PBS is phosphate buffered saline pH 7.2, C1H is FITC-Campath.

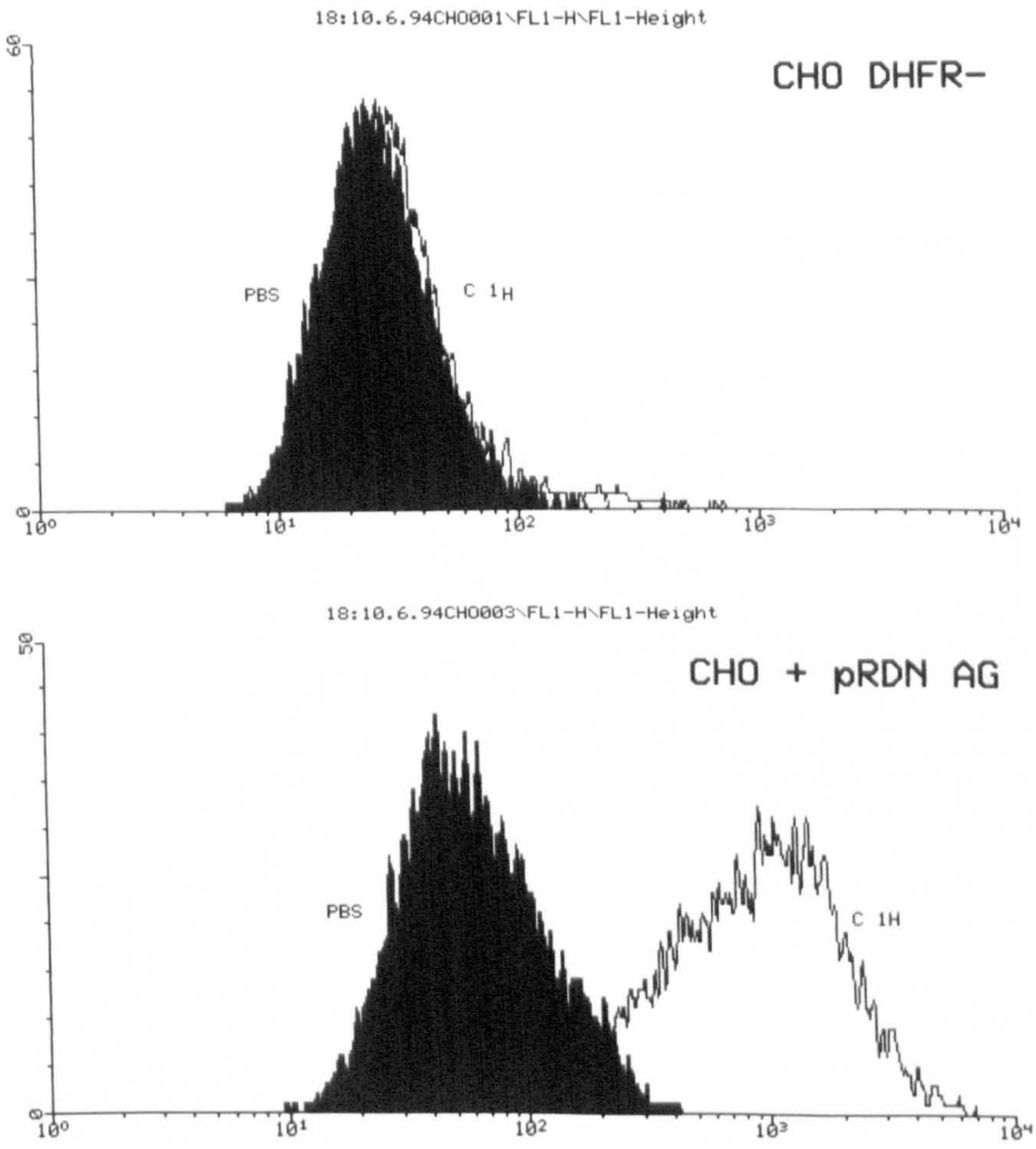
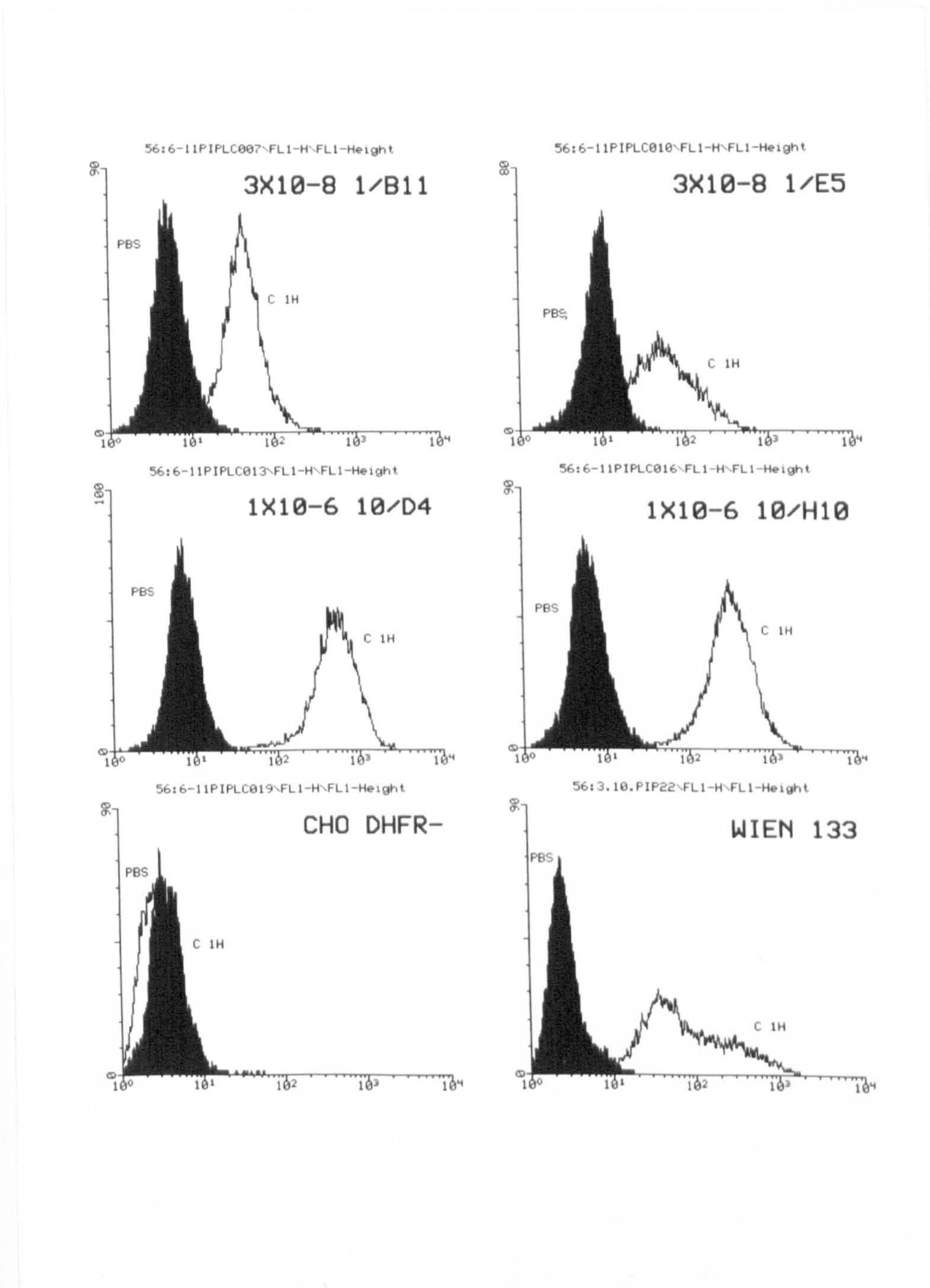


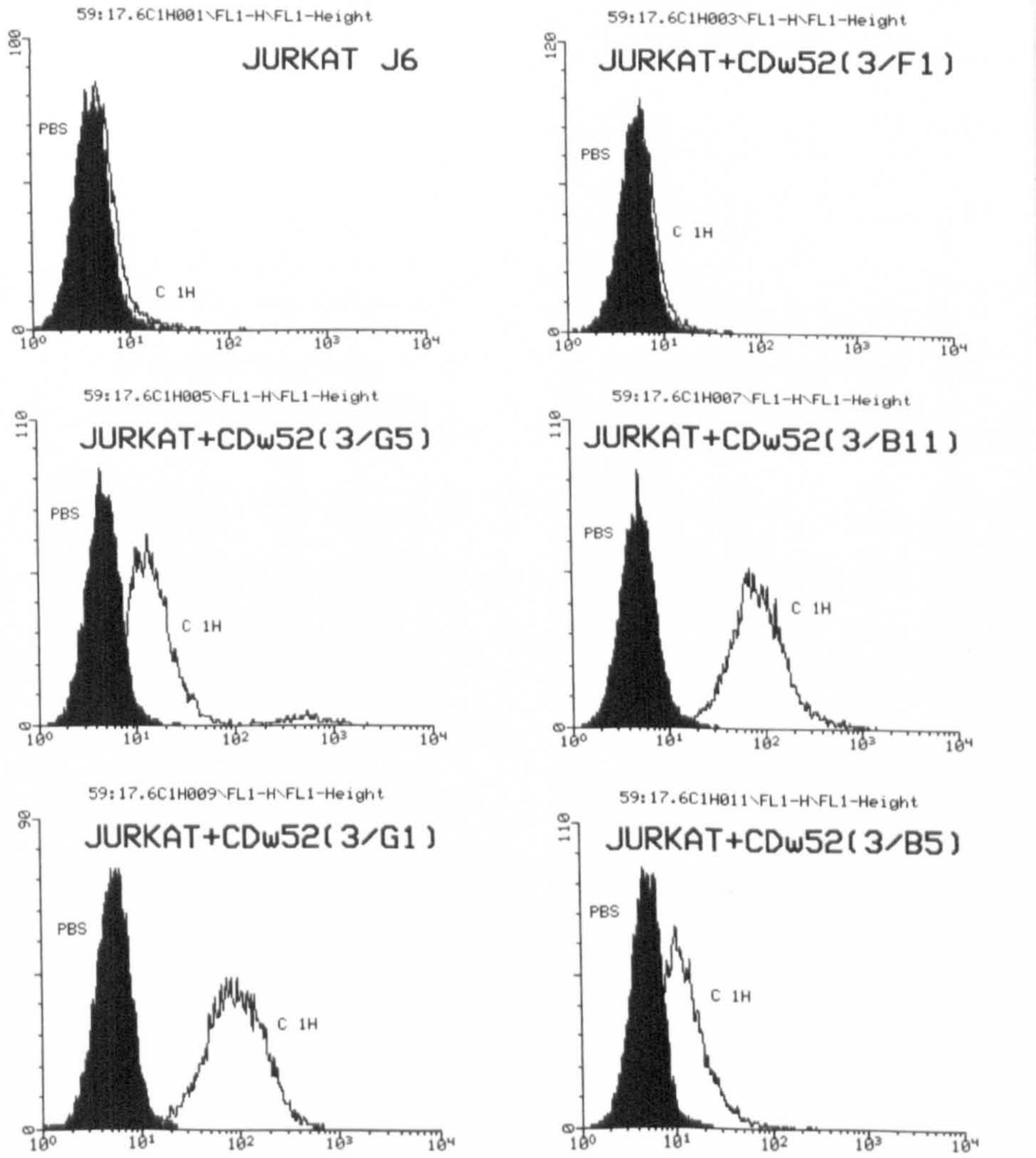
Figure 5.5 A FACscan of four $3x10^{-8}$ M MX clones (1/B11, 1/E5, 10/D4 and 10/H10) of CHO dhfr- cells transfected with the plasmid pRDN AG, parental CHO cells and Wien 133 cells for comparison. 100 µl of 10^5 cells per ml were stained with either 10 µl of a 1 in 10 dilution of FITC-Campath-1H or PBS. PBS is phosphate buffered saline pH 7.2, C1H is FITC-Campath.



5.4.2 Transfection into the Jurkat J6 cell line

Jurkat J6 T cells which had been transfected with pRDN cAG#35 were cultured in medium supplemented with 500 µg/ml of geneticin G418 (Gibco). The resulting cell line was analysed for expression by FACS analysis and the pooled cell lines were later dilution cloned. All of the individual colonies arising from the 3 cells/well plate were characterised for expression and one each of high (3/B11), low (3/F1) and two of intermediate expression (3/G5 or 3/B5) were maintained for further experimentation alongside the parental untransfected line (Fig.5.6).

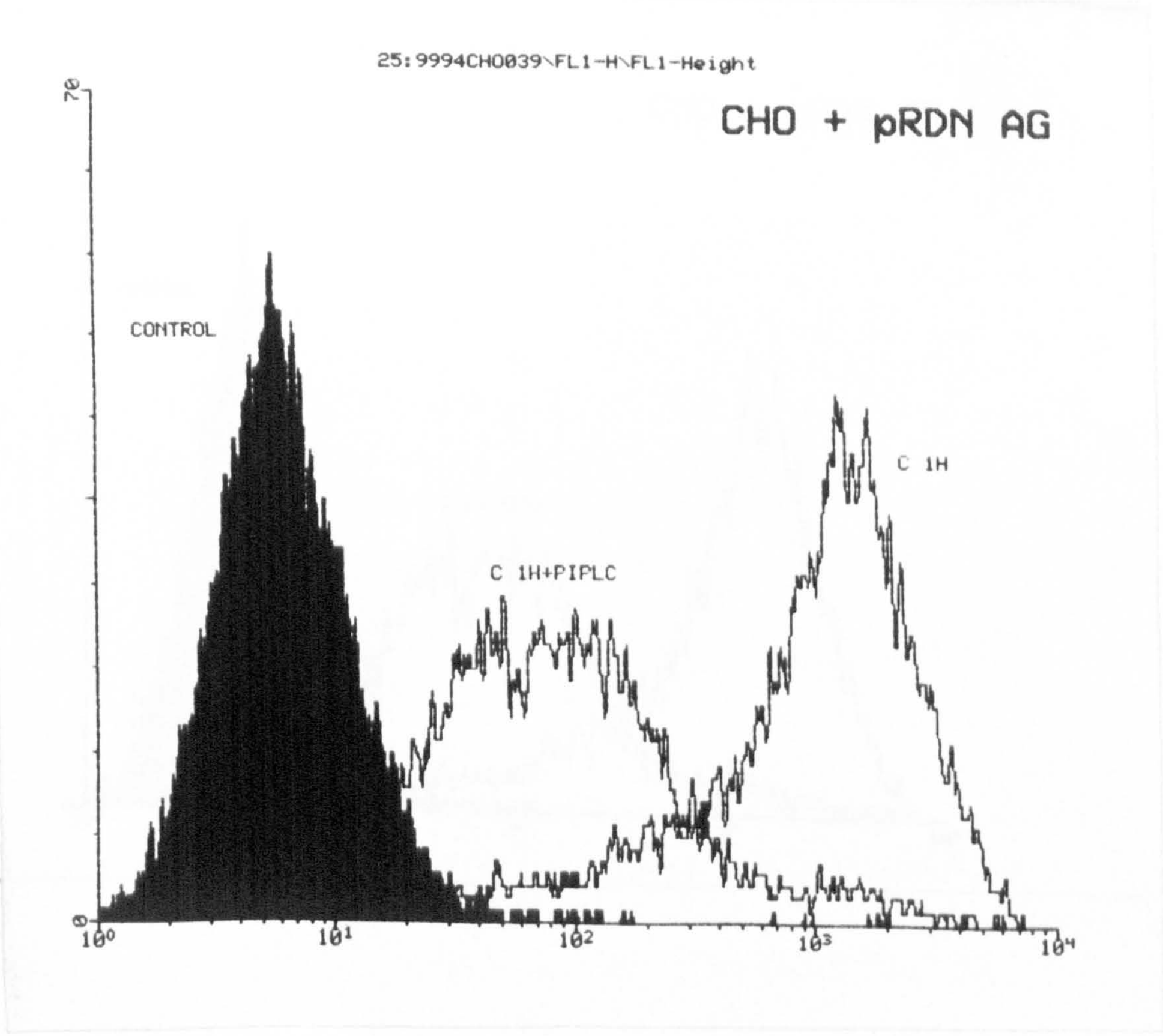
Figure 5.6 A FACscan of five clones (3/F1, 3/G5, 3/B11, 3/G1 and 3/B5) of Jurkat J6 cells transfected with pRDN AG plus parental Jurkat cells for comparison. 100 µl of 10^5 cells per ml were stained with either 10 µl of a 1 in 10 dilution of FITC-Campath-1H or PBS. PBS is phosphate buffered saline pH 7.2, C1H is FITC-Campath.



5.4.3 The confirmation of GPI-anchorage by PIPLC treatment of the transfected CHO cell line 10/D4

To confirm that the expressed antigen was GPI-anchored, enzyme assays using PIPLC (Boehringer Mannheim) were performed. PIPLC specifically cleaves the GPI-anchor directly above the site of lipid attachment to the cell. FACS data (Fig.5.7) involving extrapolation from the mean fluorescence indices on the X-axis, indicated that approximately 80% of the antigen was cleaved from $3x10^5$ cells by treatment with 10 units of PIPLC (308 units/200 µl) whilst the remaining 20% remained un-touched. This was also the case for Wien 133 cells (data not shown). It has been proposed that this is probably due to the presence of fatty acid attached to the inositol ring of some of the GPI-anchors which sterically blocks the site of cleavage (Treumann et al 1995).

Figure 5.7 A FACscan analysis of CHO cells transfected with the plasmid pRDN AG. Two of three 150 µl aliquots of cells at $2x10^6$ per ml were pre-treated with 10 units PIPLC for 90 minutes at 37°C. All three were washed in PBS prior to staining with either 10 µl of a 1 in 10 dilution of FITC-Campath-1H or PBS (control). PBS is phosphate buffered saline pH 7.2, C1H is FITC-Campath, PIPLC is phosphoinositoyl phospholipase C.



5.4.4 The cloning of the alternative sequence of CDw52

During this work two CDw52 negative variants of the Wien 133 cell line became available. The MR4 line had lost CDw52 surface expression after exposure to monocytes and was able to evade killing via the ADCC mechanism (see Chapter three, section 3.7) and the CV4 line was similarly raised by Wendy Rowan (Dept. of Cell Biology) in response to crosslinking with Campath-1H antibody (Rowan et al 1998). The generation of these lines was of interest as it suggested that patients receiving therapeutic injections of Campath-1H could possibly develop antigen negative cells. To investigate further, the cDNA from MR4 and CV4 cells was used as a source of CDw52 template for PCR experiments in which, as with the parental Wien 133 cells, the CDw52 cDNA was isolated, cloned into pUC vectors and plasmid DNA from the resulting colonies sequenced (Table 5.1).

Table 5.1 The results of sequencing cloned CDw52 PCR inserts from either MR4, CV4 or Wien 133 cDNA

Cell type	**CDw52 sequence**				**Alternative sequence**			
	Expt.1	Expt.2	Expt.3	total %	Expt.1	Expt.2	Expt.3	total %
Wien 133	1 of 2	4 of 8	2 of 3	53.8	1 of 2	4 of 8	1 of 3	46.2
MR4	1 of 5	4 of 8	N/A	38.5	4 of 5	4 of 8	N/A	61.5
CV4	2 of 6	2 of 8	N/A	28.6	4 of 6	6 of 8	N/A	71.4

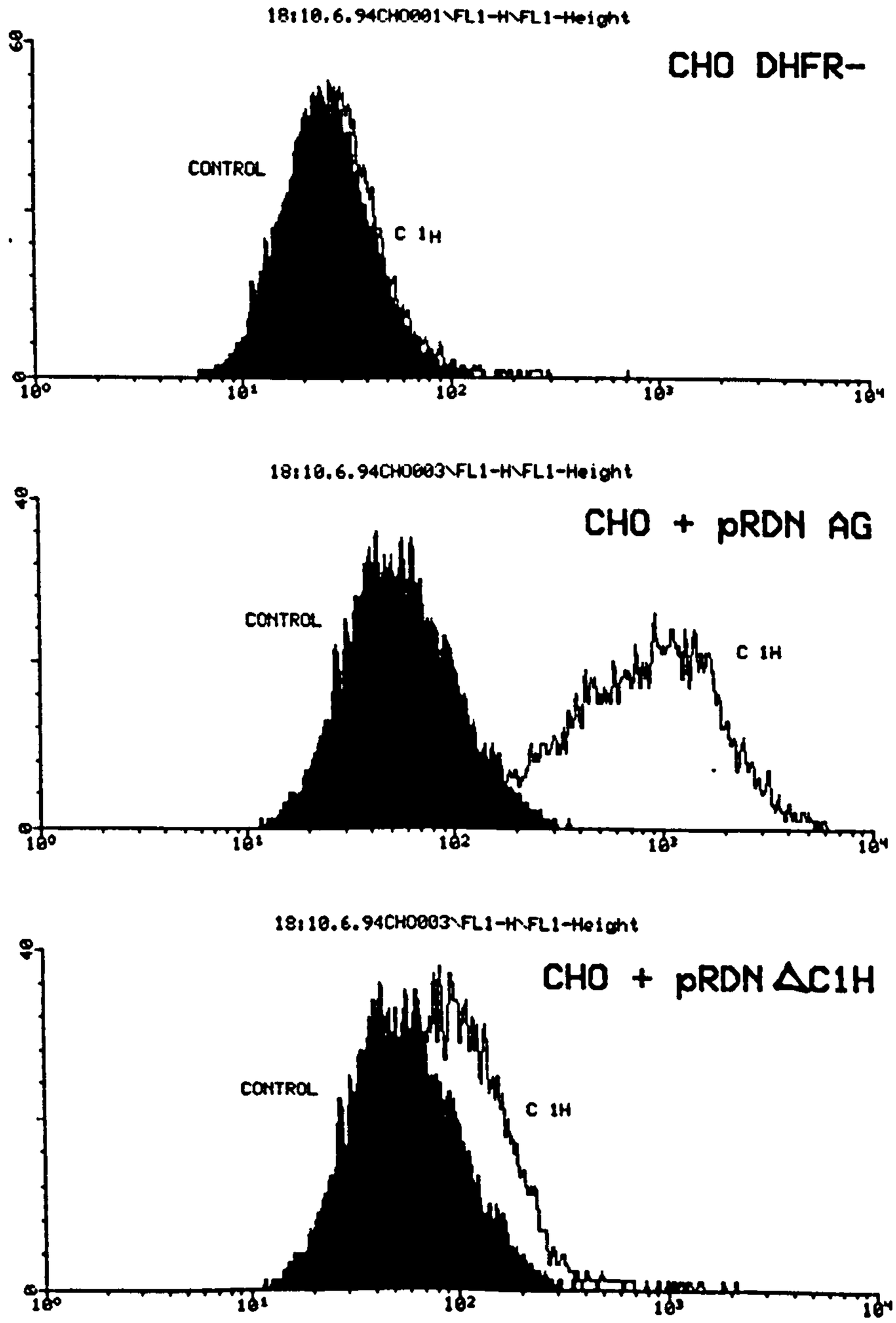
Table 5.1 shows that in the both the MR4 and CV4 lines there is a diversion away from the 53.8% distribution of the DNA sequences detected in the parental Wien 133 cells towards a higher ratio of the alternative sequence, although statistically this is not significant. Experiments investigating the comparison of both forms of CDw52 will be now be discussed.

5.4.5 Cloning and expression of the alternative CDw52 sequence (C1H) in CHO dhfr$^-$ cells

When CDw52-negative CV4 cells were injected into nude mice, the expression of CDw52 reappeared (Rowan et al 1998). To investigate whether this phenomenon was related to switches in gene expression between the two forms of CDw52, cDNA encoding the alternative sequence of CDw52 was isolated from the original PCR-derived clones and recloned into the pRDN-1 expression vector. The plasmid was named pRDN ΔC1H. Transfections into fresh CHO dhfr$^-$ cells with both the authentic and alternative cDNAs were performed simultaneously and the cells passaged through the selection procedure up to a concentration of $3x10^{-8}$ M MX. The pools of cells were then analysed by FACS

utilising FITC-labelled Campath-1H as the detecting reagent. Fig.5.8 shows that the untransfected parental cells exhibit no background staining and cells transfected with pRDN AG developed high levels of surface expression. However, the cells transfected with pRDN ΔC1H appear to have very low staining. This observation was confirmed by repeating the experiment several times.

Figure 5.8 A CDw52 FACscan of untransfected parental CHO cells or CHO cells transfected with either 1 µg pRDN AG or pRDN ΔC1H and cultured in $3x10^{-8}$ M MX. 100 µl of 10^5 cells per ml were stained with either 10 µl of a 1 in 10 dilution of FITC-Campath-1H or PBS. PBS is phosphate buffered saline pH 7.2, C1H is FITC-Campath.



There were a number of possible explanations for this observation including:- 1) the alternative cDNA encoded a form of CDw52 that was not anchored to the surface by a GPI-anchor; 2) due to the sequence change in the cDNA, the anchor was attached to a residue different from that in the authentic antigen, (both 1 and 2 would lead to the loss of detection by FITC-Campath-1H because of the loss of the antigenic epitope); 3) the antigen was not being transported to the cell surface; 4) the alternative form was either poorly or not translated from the RNA. Experiments were designed to investigate some of these possibilities.

5.4.6 Morphological differences observed in the transfected cells

The transfected CHO cells were found to grow at different rates from each other and the parental untransfected line. When cell cultures of the same cell numbers were set up and compared with time, the CHO cells harbouring pRDN AG grew at a much faster rate between sub-cultures compared to their untransfected counterpart. The reverse was seen for the cells transfected with pRDN ΔC1H. When the cells were examined microscopically morphological differences were seen (Fig.5.9). Panel A shows the untransfected CHO dhfr- cells which have a uniform packing arrangement. Panel B shows cells transfected with pRDN AG, here the cultures take on the appearance of densely packed spindle shaped cells forming an overall swirling pattern. Panel C is of the pRDN ΔC1H transfected CHO cells which demonstrate a box shaped phenotype with a disorganised distribution.

5.4.7 The growth of untransfected CHO cells or CHO cells transfected with pRDN AG or pRDN ΔC1H in nude mice

To assess whether the cells expressing one or other of the two forms of CDw52 cDNA possessed a selective biological advantage, aliquots of either $2x10^7$ cells/ml parental CHO dhfr$^-$ cells or $2x10^7$ cells/ml CHO cells transfected with pRDN AG or pRDN ΔC1H were harvested from culture. A sample was analysed for CDw52 expression by FACS staining with FITC-Campath-1H to confirm phenotype, and the remaining cells were mixed one to one with 6 mg/ml matrigel and 0.1 ml volumes injected intradermally into nude mice, 10 per group. This experiment was performed by Peter Topley and Rob Shaw (Dept of Cell Biology). After several weeks some of the mice developed tumours and these were excised and the cells teased into single cell suspensions for CDw52 FACS analysis.

Figure 5.9 The morphological differences of CHO cells transfected with the two different plasmids. The observed differences between either the regular packing of untransfected CHO cells (A), the swirling pattern of cells transfected with the plasmid pRDN AG (B) or the disorganised arrangement of cells transfected with the plasmid pRDN ΔC1H (C).

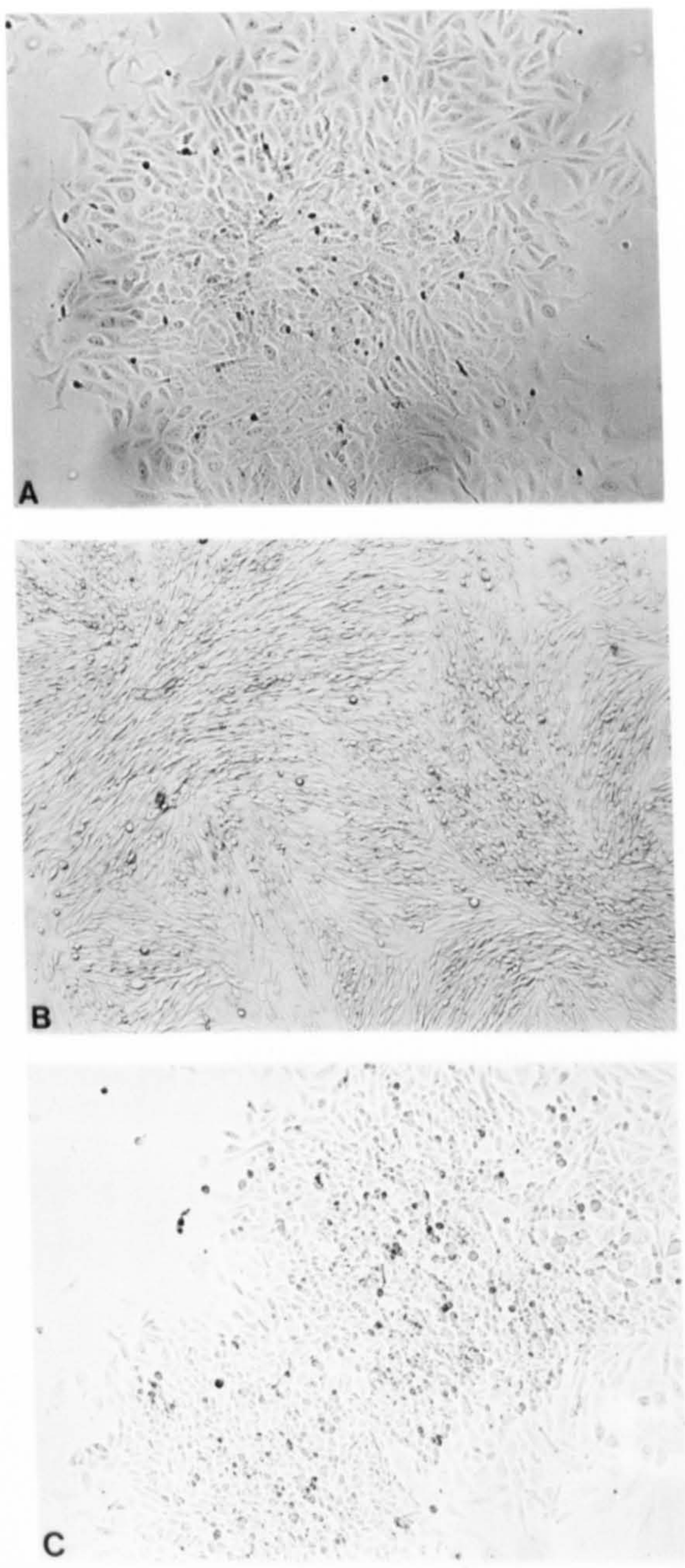


Table 5.2 The numbers of tumours arising from nude mice injected intra dermally with untransfected CHO cells or CHO cells transfected with either pRDN AG or pRDN ΔC1H

cell type	experiment 1	experiment 2	total
CHO	0 out of 10	0 out of 10	0 out of 20
CHO/pRDN AG	4 out of 10	3 out of 10	7 out of 20
CHO/pRDNΔC1H	0 out of 10	0 out of 10	0 out of 20

Only mice receiving CHO cells transfected with pRDN AG developed tumours (Table 5.2). FACS analysis of cells from excised tumours demonstrated high CDw52 expression. These results suggest that there is indeed a biological function associated with the expression of CDw52 which could perhaps either act as an adhesion molecule which allows the transfected cells to bind to some unknown ligand in vivo, or that the antigen acts as a protective molecule similar to another GPI-anchored antigen, DAF (Funabashi et al 1994).

5.4.8 ^{3}H Ethanolamine labelling of the CDw52 GPI-anchor in parental and transfected CHO cells

In an attempt to demonstrate whether the two expressed forms of CDw52 had similar GPI-anchors, cell cultures of either the untransfected CHO dhfr^{-} cells or the two transfected lines were exposed to ^{3}H Ethanolamine for eighteen hours as described in Materials and Methods. Pellets containing equivalent numbers of labelled cells ($2x10^{5}$) were lysed and samples of each lysate subjected to SDS-PAGE. Despite overloading of the sample containing cells transfected with pRDN ΔC1H, the only evidence of ethanolamine labelling of a protein of 20-28 kD appeared in the lane containing cells transfected with pRDN AG (Fig.5.10). The smeared effect is due to the large carbohydrate moiety attached to the small protein backbone. The untransfected CHO cells appeared to also have another, unknown, form of a GPI-anchored molecule of about 70 kD which acted as an internal control for loading comparisons.

5.4.9 CDw52 RNA protection assays using parental and transfected CHO cells

RNA protection studies were devised to analyse the levels of CDw52 RNA expression in the different cell lines. Equivalent numbers of cells from parental or transfected cells were pelletted and lysed and dilutions of the resulting lysates mixed with single stranded CDw52-encoding run-on probes of either forward or reverse orientation. After mixing and overnight incubation to allow annealing to occur, the annealed products were exposed to RNA-ases in order to digest unbound probe and mRNA. The anticipated remaining protected products were then separated on 6% sequencing gels which were later dried onto paper and autoradiographed overnight. Products of approximately the correct size of 206bp were detected in both types of transfected CHO cells at 10^{7}/ml and fainter bands of the same size are apparent in the 10^{6}/ml lanes (Fig.5.11). The data indicate that CHO cells are able to transcribe both forms of CDw52 cDNA and that the lack of expression associated with pRDN ΔC1H might be due to problems related to the translation or transport of the product.

Figure 5.10 A typical 15% SDS-polyacrylamide gel demonstrating Ethanolamine labelled lysates from parental CHO cells or CHO cells transfected with either the plasmid pRDN AG or pRDN _C1H. Cells were incubated for 18 hours with ^{3}H Ethanolamine prior to lysis and electrophoresis. The lanes are 1, ^{14}C protein molecular weight markers. 3, The lysate of $2x10^5$ parental CHO cells. 5, The lysate of $2x10^5$ CHO cells transfected with the plasmid pRDN AG. 7, the lysate of $2x10^5$ CHO cells transfected with the plasmid pRDN ΔC1H. Lanes 2,4,6 8 and 9 blank. The antigen CDw52 demonstrates a molecular weight of between 20-28 kD due to glycosylation.

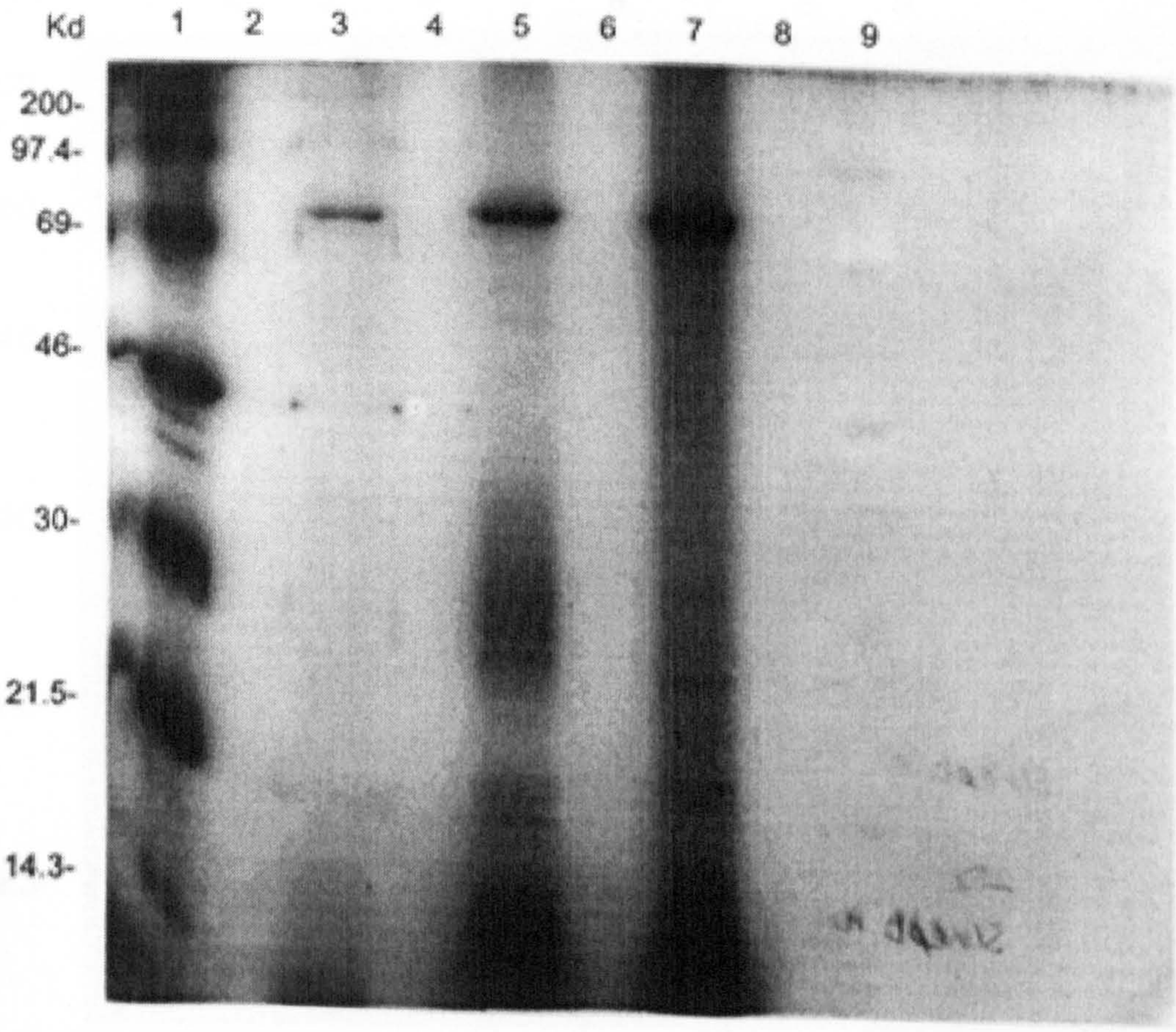
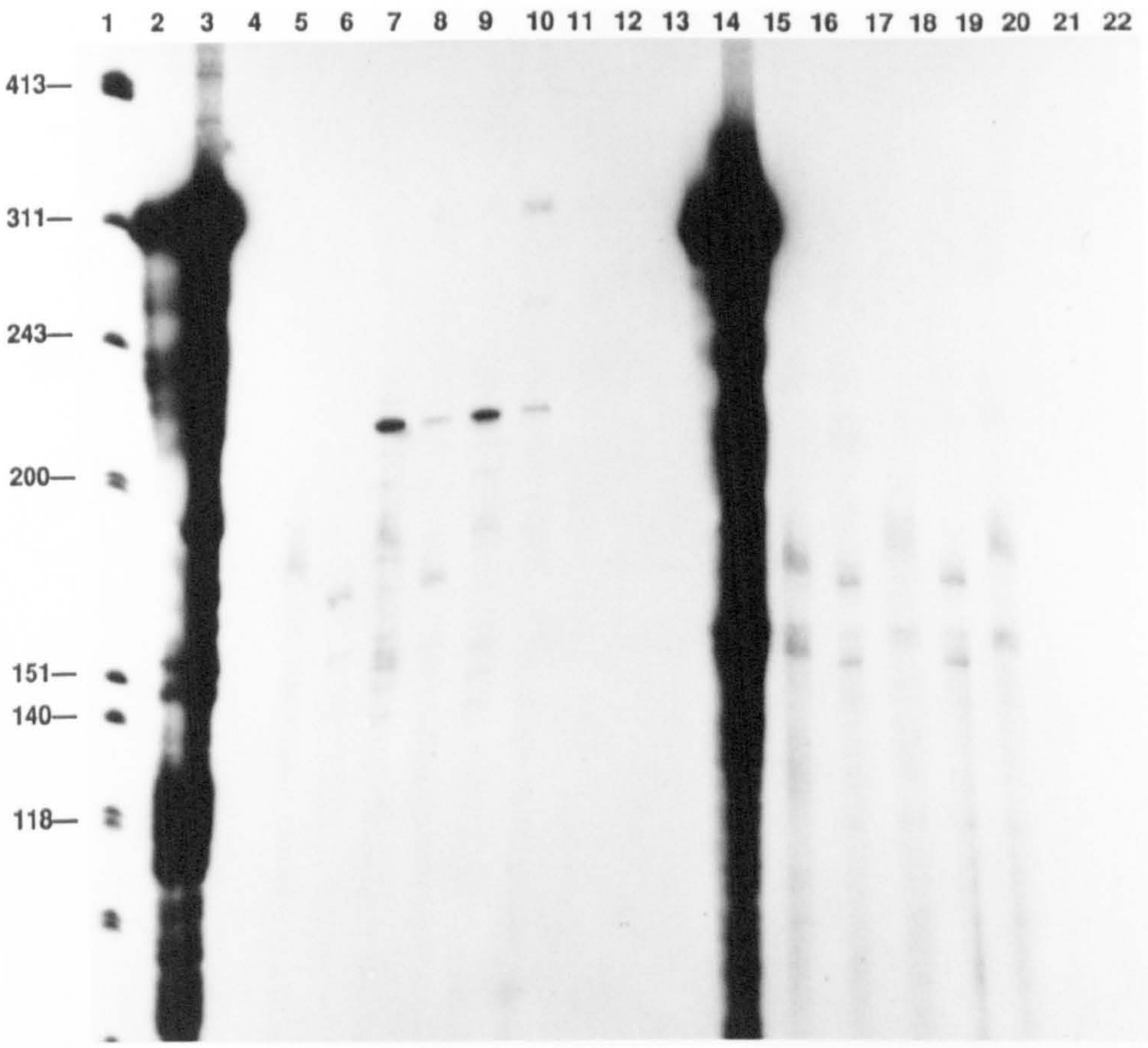


Figure 5.11 A CDw52 RNAase protection gel. CHO cells transfected with pRDN AG or pRDN ΔC1H were used as template for RNA protection studies, either 10^7 cells/ml or 10^6 cells/ml were incubated with Campath antigen probes transcribed in forward or reverse orientation in the Bluescript vector KS+ (10^5 cpm per reaction). Lane 1, ^{32}P end labelled ϕ174 marker cut with *Hinf*. Lane 3, 5 µl reverse probe; lanes 5-10, cell lysates plus reverse probe digested with RNAase of which lane 5 is 10^7/ml CHO cells, lane 6 10^6/ml CHO cells, lane 7 10^7/ml CHO cells with pRDN AG, lane 8 10^6/ml CHO cells with pRDN AG, lane 9 10^7/ml CHO cells with pRDN ΔC1H, lane 10 10^6/ml CHO cells with pRDN ΔC1H. Lanes 2,4 11-13 blank. Lane 14 5 µl forward probe, lanes 15-20, cell lysates plus forward probe digested with RNAase of which lane 15 is 10^7/ml CHO cells, lane 16 10^6/ml CHO cells, lane 17 10^7/ml CHO cells with pRDN AG, lane 18 10^6/ml CHO cells with pRDN AG, lane 19 10^7/ml CHO cells with pRDN ΔC1H, lane 20 10^6/ml CHO cells with pRDN ΔC1H. Lanes 21 and 22 blank.

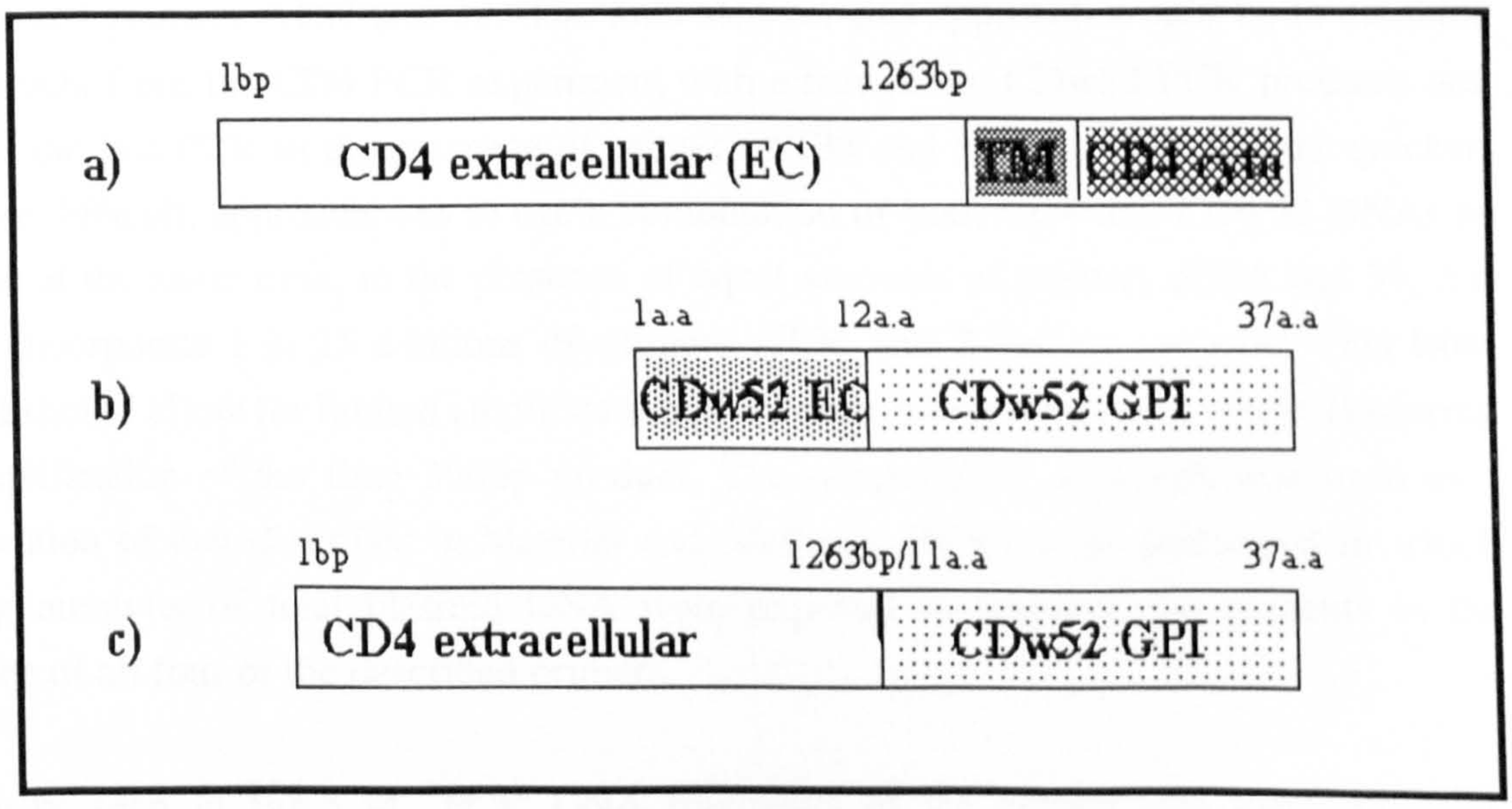


5.5 The CD4/CDw52 chimeric strategy

To overcome the technical problem of using a single CDw52 detection reagent (Campath antibody) to recognise the variant region of CDw52, a new strategy was required. A series of procedures were devised to fuse a small extracellular 5' portion of CD4, from plasmid

pT4B, in frame to the 3' ends of the two CDw52 cDNA molecules. The full upstream extracellular region of CD4 could then be recreated by straight forward cloning of the chimeric molecule as a *Sau I / Bam HI* DNA fragment. The different chimeric forms and parental CD4 could then be cloned into pRDN-1 for expression in mammalian cells. The molecule CD4 was chosen primarily for three reasons 1) it was readily available; 2) there were several detection antibodies of different specificities available commercially; 3) it was a large surface antigen not expressed on CHO cells. Within both of the cDNAs for CD4 and CDw52 there were sequences for proline residues at suitable positions for in-frame fusion. The proline in the CD4 extracellular region was at base 1263 just upstream to the membrane spanning domain and in CDw52 the proline residue was at amino acid position 11, just one amino acid upstream to the GPI-anchor attachment site.

Figure 5.12 A diagrammatic representation of the polypeptides and the regions of interest. The top one a) is CD4, the middle one b) is CDw52 and the bottom one c) is the CD4/CDw52 chimera. The portions mentioned are the CD4 and CDw52 extracellular regions (EC), the CD4 transmembrane region (TM), the CD4 cytoplasmic tail (cyto) and the CDw52 GPI-anchor. The numbers represent either the base or amino acid number.



Four 30-31mer DNA primers were designed as shown in Fig.5.13. The first forward primer (AT 35) exactly matched part of the 5' end of CD4 cDNA amino acids 1009-1038 and incorporated both *Hind III* and *HpH I* sites for cloning purposes. The second primer, a reverse primer (AT 36), was complementary to the CDw52 3' coding strand residues 15-11 and was also complementary to the 3' end of the extracellular coding strand of the CD4, bases 1263-1245. Primer number three (AT 37), another forward primer, was the coding equivalent of primer number two and matched the 3' extracellular portion of CD4 (bases 1245 - 1263) and the overlap into CDw52 (amino acids 11 - 15). A fourth primer (AT 39), was similar to the reverse primer #3684 used to originally isolate the CDw52 cDNAs. This reverse primer was complementary to the coding region containing

the stop signal in the 3' end of CDw52 (common to both forms of CDw52) amino acid numbers 38-34, plus *Bam HI* and *Eco RI* cloning sites.

Figure 5.13 The PCR primers used to form the in frame fusion of extracellular CD4 to the CDw52 GPI-anchors. The underlined bases are those of CD4 origin. Those in bold type represent restriction sites.

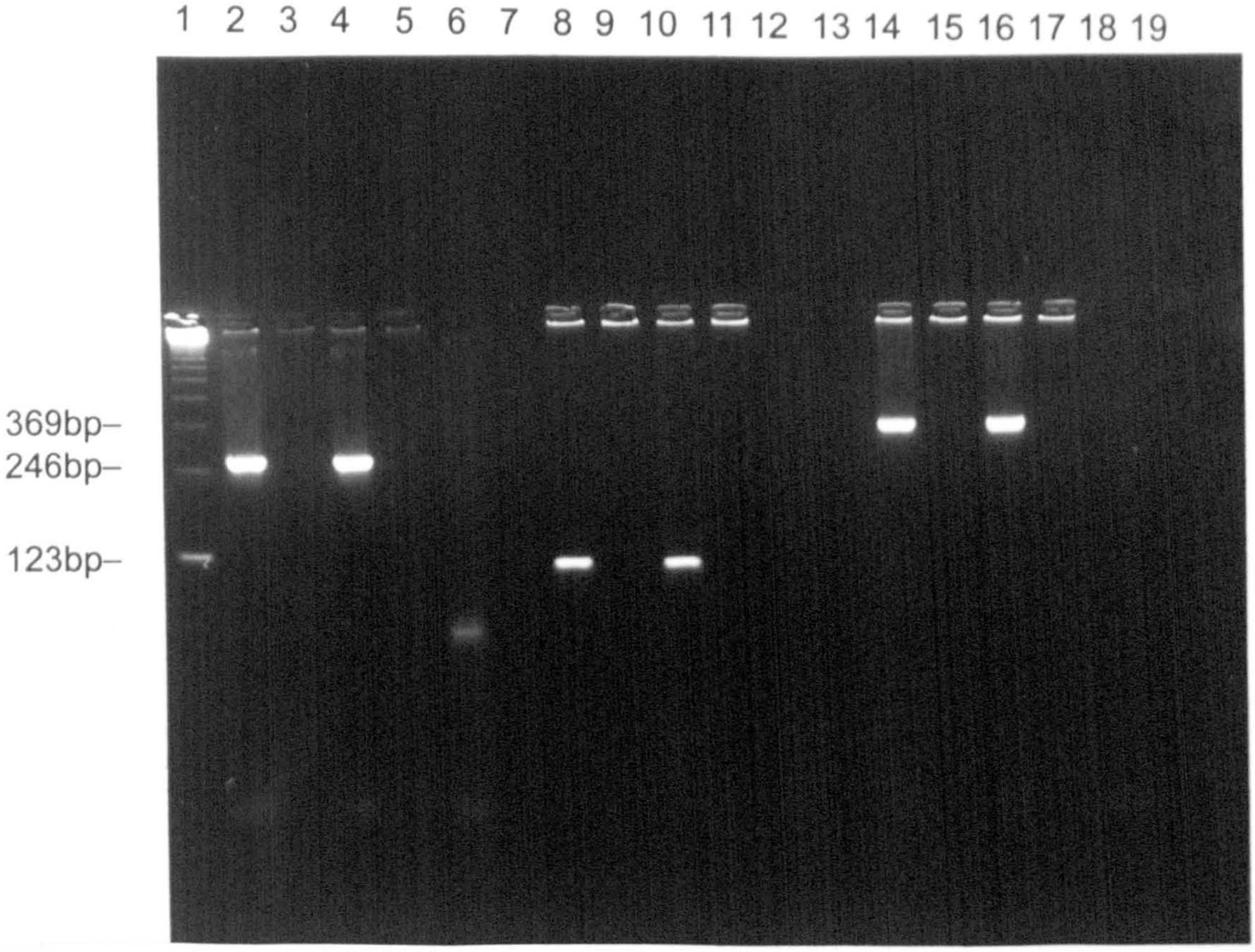
AT 35 a 30-mer CD4 primer	5' GTG**AAGCTT**GTGGTGATGAGAGCCACTCAG 3'
AT 36 a 30-mer CDw52 and CD4 primer	5' GCTGGATGCTGAGGGCTGCACCGGGGTGGA 3'
AT 37 a 30-mer CD4 and CDw52 primer	5' TCCACCCCGGTGCAGCCCTCAGCATCCAGC 3'
AT 39 a 31-mer CDw52 primer	5' GATC**GGATCCGAATTC**TCAACTGAAGCAGAA 3'

To form the fusion between the CD4 extracellular region and either of the two CDw52 tails using PCR, two approaches were feasible. One approach was to perform three PCR reactions concurrently to obtain either the desired 286bp CD4 region with CD4 plasmid pT4B DNA as template and utilising primers AT 35 and 36, or to obtain the two relevant 110bp CDw52 tail portions utilising CDw52 plasmids pRDN AG or pRDN ΔC1H DNA as template and primers AT37 and 39. The final step for this approach would be to combine the products from the CD4 PCR experiment with either of the CDw52 PCR products and perform the last PCR in the presence of primers AT35 and 39 only. The second quicker, but more difficult, approach was to use a combination of both CD4 and CDw52 DNAs as template at the same time, in the presence of equal amounts of primers AT35 and 39, but also to incorporate 1 in 25 dilutions of primers AT36 and 37 in the reaction. This latter method should allow for limited amplification of the intermediate products whilst favouring the amplification of the final 390bp product. The second PCR approach was used as a modification of that described in Material and Methods. PCRs were performed in which 100 ng amounts of total plasmid DNA were exposed to amplification reagents in the presence of all four of the described primers.

As can be seen in Fig.5.14, PCR DNA fragments of the correct size were obtained, purified and cleaved with the relevant restriction enzymes prior to cloning into pUC vectors as *Hind III / Bam HI* inserts (data not shown). Several of the resulting colonies were used to prepare mini preparations of plasmid DNA and these were sequenced in both orientations to confirm the sequence. Nearly all of the DNA samples tested were correct (data not shown). DNA preparations from selected colonies were scaled up to maxi preparation scale and the DNA retrieved on caesium gradients. Cleavage of the resulting DNA with both *Sau I* and *Bam HI* generated DNA fragments of 200 bp. (data not shown). The fragments were cloned into pT4B which had been cleaved with the same enzymes to remove the portion of CD4 corresponding to the end of the extracellular region, the

transmembrane region and the cytoplasmic region. This resulted in the reconstruction of the full extracellular CD4 region now bearing CDw52 GPI-anchor signals.

Figure 5.14 CD4/CDw52 PCR gel in which duplicate products of the correct size can be seen. Lane 1 123bp markers, lanes 2 and 4 286bp PCR products from pT4B with primers AT 35 and 36, lanes 3 and 5 mock reaction of pT4B lane minus primers, lanes 8 and 10 110 bp product from pRDN AG with primers AT 37 and 39, lanes 9 and 11 mock reaction of pRDN AG minus primers, lanes 14 and 16 390bp product of both pT4B and pRDN AG with primers AT 35,36, 37 and 39, lanes 15 and 17 mock reactions of pT4B and pRDN AG minus primers. Lanes 6,12 and 18 water controls minus DNA but with primers lanes 7,13 and 19 water controls minus DNA and primers. In lane 6 (a water control) there is a non-specific <100bp PCR product.



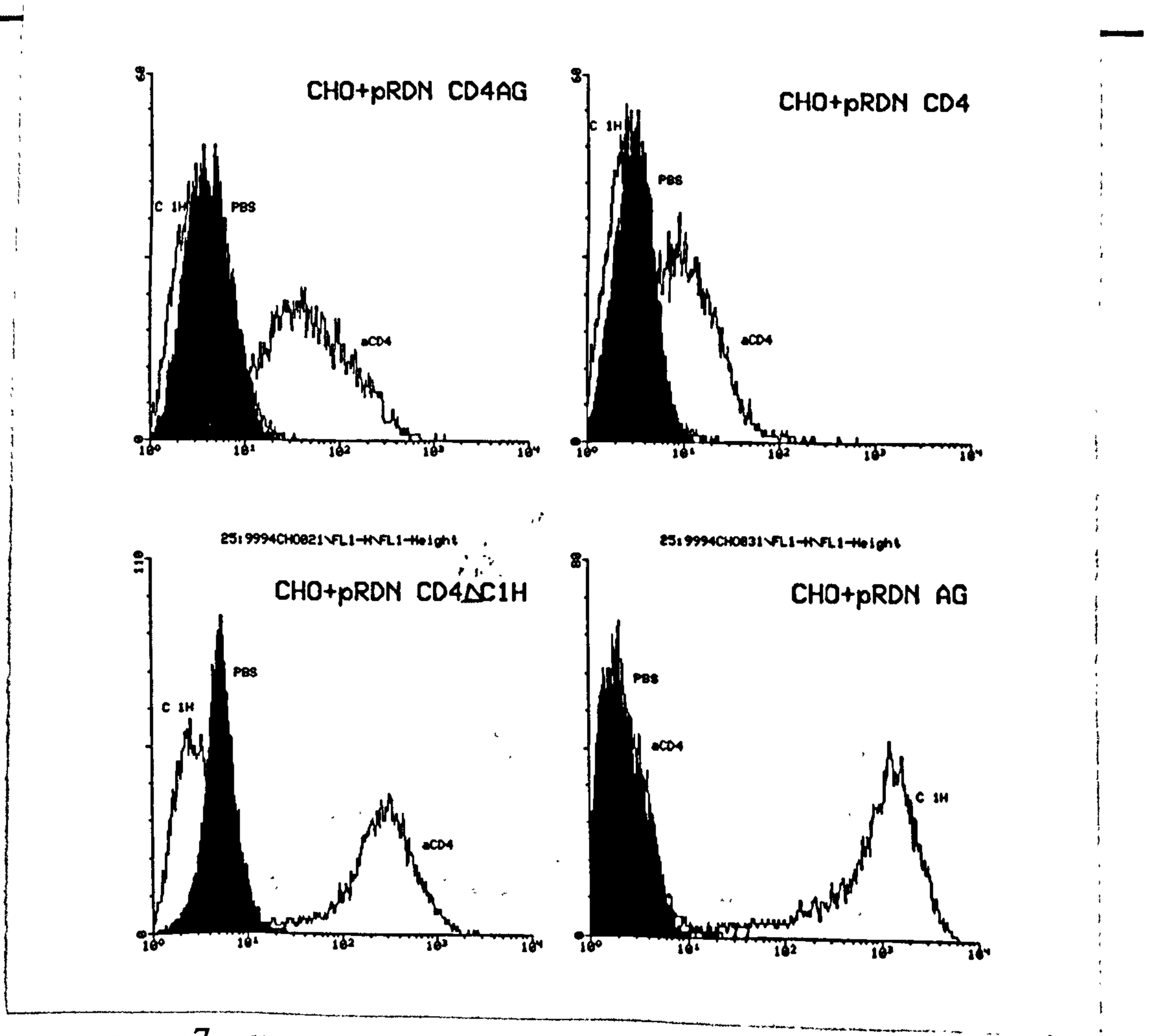
To compare the expression of the two CD4/CDw52 chimeras, DNA encoding both genes was cleaved from the modified T4B using *Eco RI* and the resulting 1362 bp DNA fragment was ligated into the similarly cleaved pRDN-1 to generate pRDN CD4/AG or pRDN CD4/ΔC1H. From the original T4B, the 2901 bp DNA fragment encoding the CD4 molecule containing the extracellular and intracellular sequences, was also ligated into pRDN-1 as an *Eco RI* fragment and the orientation checked by restriction enzyme analysis (results not shown). A plasmid with the correct sequence was named pRDN CD4. Plasmids pRDN AG and pRDN ΔC1H were already available as controls.

5.5.1 The transfection of DNA encoding CD4/CDw52 chimeras, CD4 or CDw52 into CHO cells

pRDN CD4/AG, pRDN CD4/ΔC1H, pRDN CD4 and pRDN AG were independently used to transfected CHO dhfr$^-$ cells and the transfected cells were gradually passaged through the

selection procedure. The selection of high expression levels was obtained by gradually increasing the MX concentration to $3x10^{-8}$ M. Throughout the amplification steps, pools of the transfected cells were analysed for both CD4 and CDw52 expression utilising FACS analysis. Once the cells were growing stably in the $3x10^{-8}$ M MX selection medium they were dilution cloned. The results of the FACS analysis of typical clones are shown in Fig.5.15. As expected cells transfected with the CD4/CDw52 chimeras lost CDw52 staining whilst the expression levels of the controls were normal. However, cells harbouring pRDN CD4/ΔC1H not only developed CD4 expression but to a level higher than that seen in the cells transfected with pRDN CD4/AG. This would indicate that the previous loss of CDw52 expression in CHO cells transfected with pRDN ΔC1H was a transport rather than translation problem. To confirm the expression levels, the same cells were utilised in CD4 immunoprecipitation experiments.

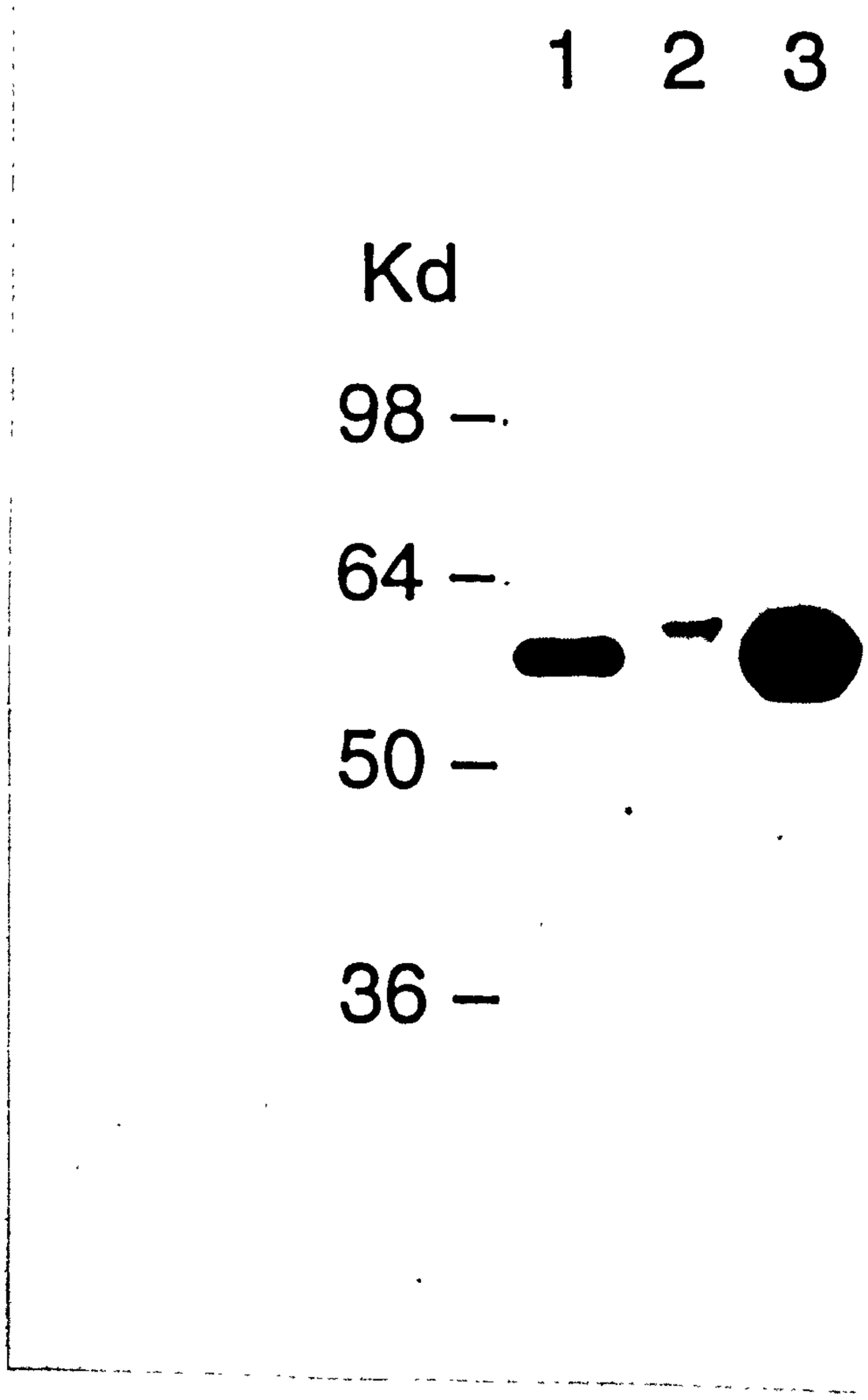
Figure 5.15 CD4 and CDw52 FACS analysis of typical transfected CHO clones. CHO cells transfected with either pRDN CD4 or pRDN AG were compared with CHO cells transfected with pRDN CD4/AG or pRDN CD4/ΔC1H for expression of CD4 or CDw52. 100 µl of 10^6 cells per ml were stained with 10 µl of a 1 in 10 dilution of either FITC-Campath-1H (C1H) or FITC-anti CD4 (a-CD4). Phosphate buffered saline pH 7.2 (PBS) was used as control.



Aliquots of $2x10^7$ cells of all of the four transfected cell lines were lysed and the lysates immunoprecipitated with 50 µl of a $F(ab')_2$ fragment of anti-CD4 antibody bound to

sepharose beads. The products were separated on 8% non-reducing SDS-polyacrylamide gels and transferred, by Western blotting, onto nitrocellulose paper for detection of the protein. Cells harbouring pRDN CD4/AG (1), pRDN CD4 (2) and pRDN CD4/ΔC1H (3) all show CD4 precipitates whilst the cells with pRDN AG (4) are negative for CD4 (Fig.5.16). The precipitates of (1) and (3) are of a smaller size than the full length CD4 (2) because of the presence of the CDw52 GPI-anchor. Cells harbouring pRDN CD4/ΔC1H have higher levels of CD4 precipitates.

Figure 5.16 A typical CD4 western blot of transfected CHO cells. Lysates from $2x10^7$ CHO cells transfected with lane 1 pRDN CD4/AG, lane 2 pRDN CD4/ΔC1H, lane 3 pRDN CD4 or lane 4 pRDN AG, were immunoprecipitated using anti-CD4 sepharose and the resulting proteins separated by SDS-PAGE. After transfer to nitrocellulose by western blotting, the filters were probed with anti-CD4 antibodies. The CD4 protein has a molecular weight of 60kD.

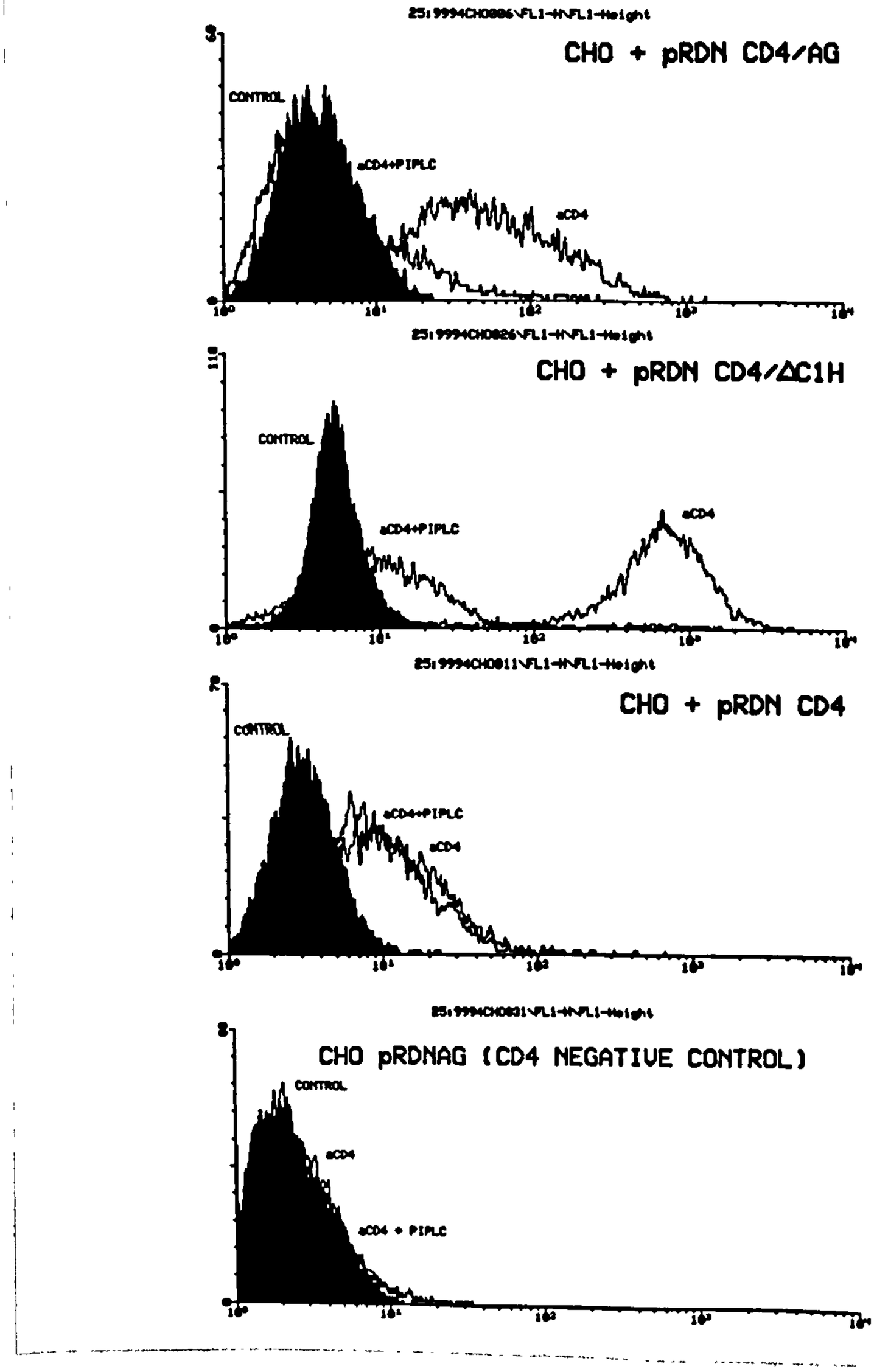


5.5.2 The treatment of the CD4/CDw52 transfected CHO cells with PIPLC

To confirm that the CD4/CDw52 chimeras have GPI-anchors, the four cell lines were exposed to PIPLC treatment. The treated cells were later FACS analysed with either FITC

labelled anti-CD4 or Campath-1H as relevant (CDw52 data not shown). PIPLC treatment of cells transfected with either pRDN CD4/AG or pRDN CD4/ΔC1H led to a decrease in the surface expression of CD4 (Fig.5.17). Cells harbouring pRDN CD4 were not affected. CHO cells transfected with the plasmid pRDN AG never expressed detectable CD4.

Figure 5.17 FACS analysis of PIPLC treated transfected CHO cells. Transfected CHO cells bearing either pRDN CD4/AG (top), pRDN CD4/ΔC1H (upper middle), pRDN CD4 (lower middle) or pRDN AG (bottom) were treated with and without PIPLC prior to staining with FITC-anti CD4 (a-CD4). Phosphate buffered saline (PBS) was used as control.

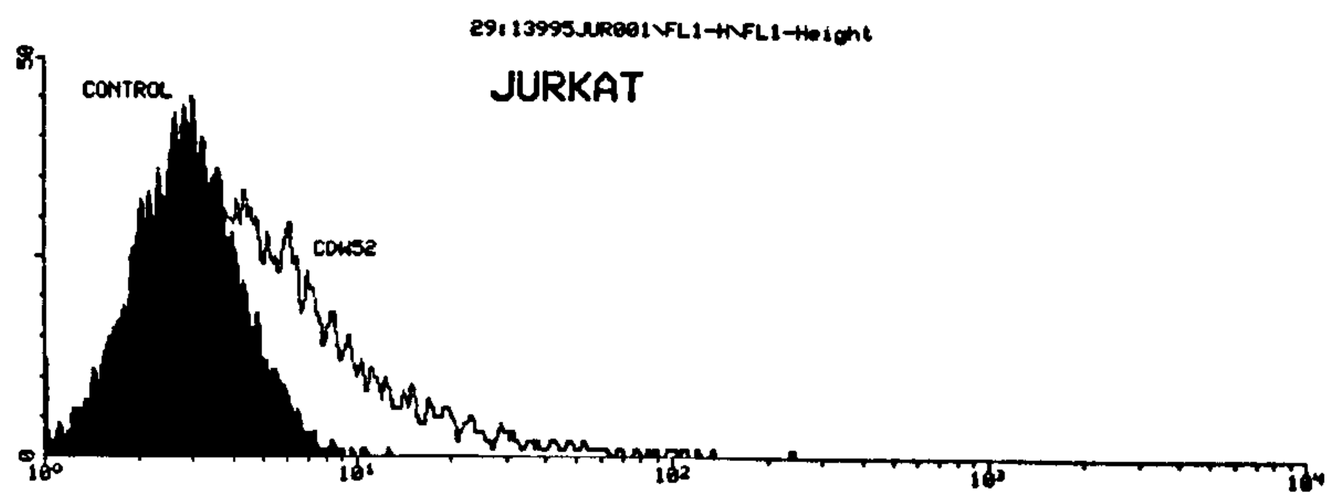


5.5.3 The tranfection of Jurkat J6 cells with pRDN AG and pRDN ΔC1H

As an independent test of whether pRDN ΔC1H could be expressed in mammalian cells, 10 μg was co-transfected with 1 μg of the p321neo plasmid, into Jurkat J6 cells. Concurrently, pRDN AG was used as control. After selection in G418 containing medium,

both cell lines were FACS analysed for CDw52 expression in comparison with the parental line utilising FITC-labelled Campath-1H. Both plasmids were able to direct CDw52 expression but with different profiles (Fig.5.18). The pRDN AG transfected cells demonstrated high expression levels of CDw52 as expected however, in contrast to the large difference in expression seen between the two variants in CHO cells, the Jurkat cells transfected with pRDN ΔCIH also showed good expression of CDw52. There was still however a quantitative difference in levels of expression observed.

Figure 5.18 CDw52 FACS analysis of transfected Jurkat J6 cells. Aliquots of 100 µl ($1x10^6$ cells per ml) of parental cells or cells transfected with pRDN AG or pRDN ΔC1H were stained with 10 µl of a 1 in 10 dilution of FITC-Campath-1H or PBS. PBS is phosphate buffered saline pH 7.2, CDw52 is Campath antigen.



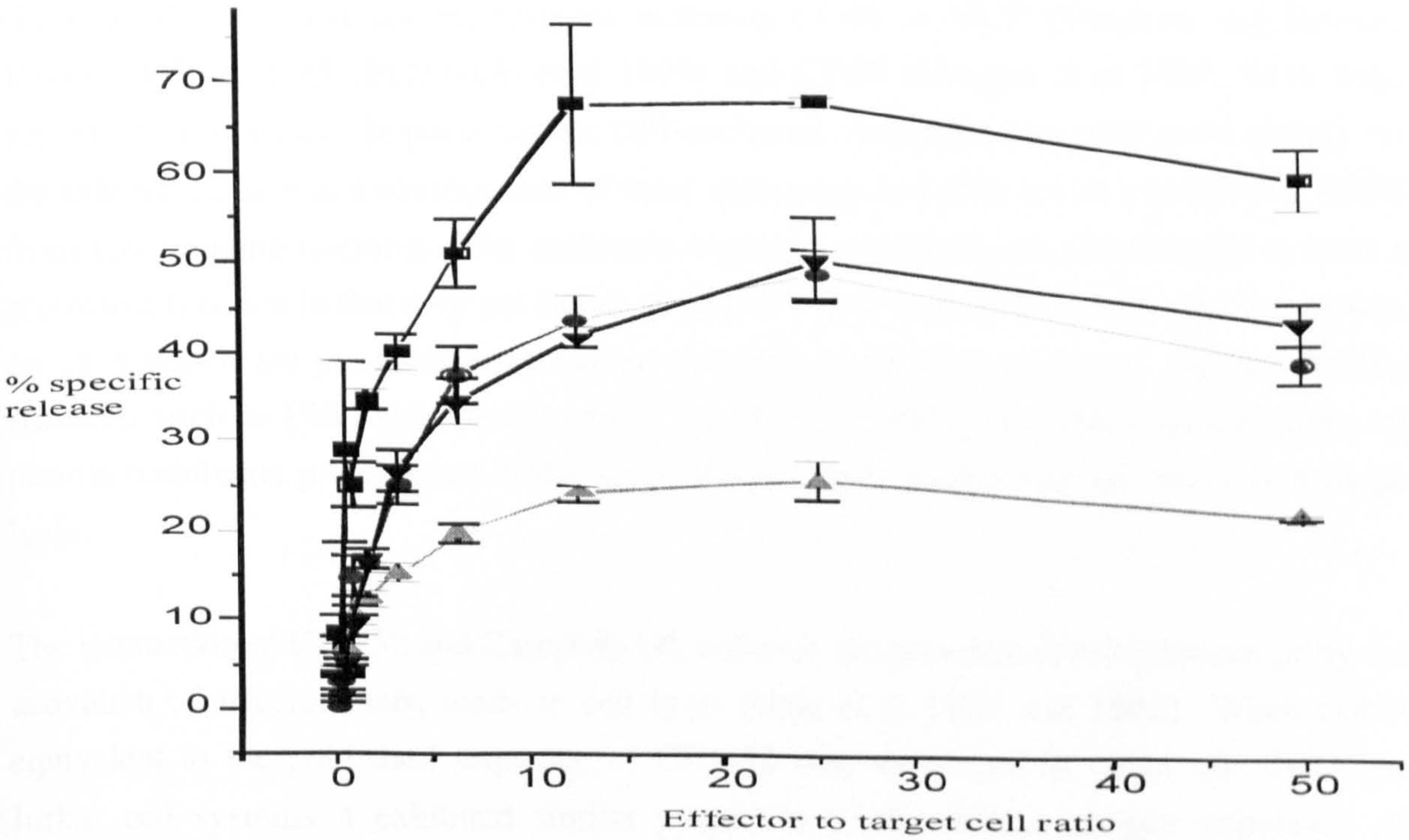
5.6 Utilisation of transfected cells

5.6.1 In ADCC assays

Clones of CHO CDw52 transfectants never proved to be reliable targets in ADCC assays due to their inability to be readily lysed in response to antibody (data not shown). However, some data was obtained using Jurkat 3B/11 transfectants as target cells (Fig.5.19). These were lysed efficiently in the presence of Campath (1 µg/ml) at all effector

to target cell ratios greater than 10:1. Unlike Wien 133 cells, moderate lysis was still observed when antibody was omitted. Conversely, untransfected Jurkat cells demonstrated some lysis in the presence of antibody which increased to that of the transfected cells when antibody was omitted. The significance of the data, if any, is unclear, but it does suggest that the presence of CDw52 might be protective against lysis from non-ADCC mechanisms. The situation needs to be clarified by studying several clones and it should be stated that the site of integration of the plasmid into each cell genome is not known and therefore the transfected cell phenotype may be altered.

Figure 5.19 The use of CDw52 transfected Jurkat cells as ADCC target cells. 10 transfected or parental Jurkat cells were used as target cells in a Campath-1H (1 µg/ml) ADCC assay at different effector to target cell ratios. Symbols represent transfected Jurkat cells with ■ or without ● Campath-1H, represents parental Jurkat cells with ▲ and without ▼ antibody. The data is plotted as mean ± standard errors.



5.6.2 Transfected Jurkat cells in crosslinking experiments

The untransfected and the pRDN AG transfected Jurkat clones were used in experiments where signalling events were monitored by crosslinking the expressed antigen (see Chapters three and four). The cells assayed had been co-transfected with the expression plasmid plus a neomycin resistance encoding plasmid and subsequently selected by culture in medium containing G418, an analogue of neomycin. Recent evidence now indicates that drugs similar to neomycin interfere with signalling events probably by blocking the Protein-Kinase C step (Lin et al 1991). As a consequence, the transfected cells were cultured minus G418 for a week prior to repeating the experiments . The Jurkat cell line is a 'T' cell line that does not express surface Ig and therefore the observed effects are due entirely to the transfected CDw52 antigen.

5.7 Discussion

The antigen CDw52 has been known since the early 1980s and has been sequenced and characterised (Xia et al 1991 and 1993b, Hale et al 1990). Extensive studies on the carbohydrate composition have been undertaken and the data published (Treumann et al 1995). The antigen has also been used as a means of activating T cells (Rowan et al 1995) and has been utilised as a therapeutic target. However despite all of this information there has been little progress on determining the function or even possible function of CDw52. The antigen is one of a significant number of molecules which are attached to the cell surface via a GPI-anchor. Other molecules which share this property have been identified. In protozoan parasites they include VSG (variant surface glycoprotein) on typanosomes (Cross 1990) and gp63 on Leishmania. In rats Thy-1 has been shown to be GPI-anchored (Fasel et al 1989) and several antigens including CD46 or MCP (Simpson and Holmes 1994), CD55 or DAF (Funabashi et al 1994) and CD59 (Morgan et al 1993) have been identified in mammals. In parasites, the GPI-anchored molecules may pack more closely on the external surface as a consequence of their anchorage and thus act as a protective shield from host immune response. The molecules found in mammals are also thought to have a protective function in that they are found on sperm in the male genital tract and are present on cells which are prone to lysis during immune attack (Simpson and Holmes 1994). Ailments such as PNH (Ueda et al 1992, Takeda et al 1993), in which GPI-anchors on plasma membrane proteins are defective or absent, leads to the loss of red cells through lysis.

The interaction of CDw52 and Campath-1H, either in the presence of complement or by the activation of effector cells, leads to cell lysis (Hale et al 1983 and 1985). When cDNA equivalent to the published sequence of CDw52 was expressed in either the CHO and Jurkat cell systems it exhibited similar properties to the natural antigen expressed on lymphocytes (Hale et al 1996). The CDw52 transfected cells proved to be amenable to experimental analysis and could be used as an alternative to isolated mononuclear cells or suspension T and B cell lines. The authentic CDw52 was recognised by Campath-1H and was shown to contain a GPI-anchor by utilising the specific enzyme PIPLC (Hale et al 1996). As with CDw52 expressed on mononuclear and Wien 133 B cells, PIPLC treatment resulted in the substantial reduction but not complete loss of antigen expression. This may be a consequence of excess palmitylation of the GPI-anchor inositol ring leading to blockage of the cleavage site on a percentage of the molecules (Treumann et al 1995).

The discovery of the second variant of CDw52 sequence was not in itself a novel finding, but the variant had previously been described as a possible PCR artefact (Xia et al 1991) and similar variant of CDw52 sequence was also described by Perry (et al 1992). Amongst

cDNA prepared from Wien 133 cells both forms of PCR product were found to be present in approximately equal proportions. When antigen negative variants of Wien 133 cells were obtained by selective pressure, the ratio altered towards higher levels of the alternative sequence. The variation from the authentic sequence was found to reside outside of the mature protein in residues 16 and 17. The resulting change in amino acids was from asparagine and isoleucine to serine and methionine respectively. These residues are in a context which is critical for determining the correct positioning of the GPI-anchor and potentially creates an alternative GPI-anchor site (Moran and Caras 1991a and b). In our investigation the alternative CDw52, C1H, was not expressed at high levels in CHO cells transfected with a plasmid encoding that sequence (Hale et al 1996). The expression of the two forms in CHO cells was demonstrated by FACS analysis and correlated with reproducible morphological differences in culture. Ethanolamine labelling of transfected cells was consistent with the low expression levels of the alternative form however, RNAase protection assays indicated that mRNA was being transcribed in these transformants. The introduction of both cell lines into nude mice revealed striking differences, in that only those mice receiving cells bearing the authentic cDNA, developed tumours.

The reason for the lack of expression in cells, detected by FACS, was postulated to be either a lack of recognition of the antigen by the antibody due to an altered epitope or loss of the epitope. The epitope for recognition by the antibody Campath-1H has been demonstrated to reside within residues 9-12 of the mature protein in addition to an undefined component of the GPI-anchor (Hale et al 1990). The anchor is added post-translationally via the serine residue at position 12. It was suggested that the alternative CDw52 might have its GPI-anchor attached to a different residue or that the antigen may no longer possess a GPI-anchor. Since the Campath-1H epitope may be modified in the alternative form of CDw52 it was unreliable to use Campath antibody as detecting regent. To overcome the technical problem, both the authentic and alternative sequences of the CDw52 GPI-anchor were cloned downstream of the extracellular sequence of CD4 cDNA in expression vectors and these were transfected into CHO cells and expressed under selection with high concentrations of MX. Surprisingly, not only were both forms expressed and recognised by anti-CD4 antibody, but both were also demonstrated to be GPI-anchored by PIPLC treatment (Hale et al 1996). Ethanolamine labelling of the resulting cells confirmed this, as did immunoprecipitation of the lysed labelled cells with anti-CD4 antibody. Both CD4/CDw52 chimeras were detected as precipitated protein of the same mobility on SDS-polyacrylamide gels. When compared to the control CD4 cDNA, which was transfected and expressed with its original transmembrane and cytoplasmic regions, the mobility of both of the GPI forms was less then the intact CD4, reflecting the addition of GPI-anchors. Subsequently, Jurkat cells transfectants were produced in which

both forms of the antigen were expressed. However, the originally described CDw52 variant was always expressed at a higher level suggesting that this form of CDw52 gene was more efficiently processed and transported to the surface.

The development of the CDw52 expressing Jurkat cell lines proved to be of considerable importance in antigen binding studies for several reasons. Firstly, in the definition of the mode of action of CHO Campath in in vitro assays (Chapter three) and for comparison of antibodies isolated from different host cells or of different isotype (Chapter four). Secondly, for assessing the functional changes in NSO antibody associated with the development of media (Chapter four). Furthermore, a number of potential uses for the CDw52 expressing CHO cell line, 10/D4, have now also emerged. Dr. R. Lifely (Wellcome, personal communication) successfully evaluated the cell line as a source of pure antigen. Prior to the development of the transfected cells, the antigen was isolated from human spleens which had been surgically removed for medical reasons and thus not in abundant supply. The purified recombinant CDw52 antigen could be used as a reagent for studying antibody responses in Campath treated patients, in assays to compare the affinity of Campath antibodies and for further studies on the structure of the antigen itself. The 10/D4 line was used to assist in the evaluation of radio-labelled Campath-1H for the treatment of solid tumours (Hutchins et al 1995).

The derivation of the CDw52 negative, CV4 and MR4, Wien 133 cells in the laboratory pre-empted the natural development of CDw52 negative cells in patients receiving Campath-1H. The use of these cells in in vitro assays helped to predict the possible consequences in patients (Chapter three, section 3.7). In the early stages of Campath therapy, antigen bearing lymphocytes are severely depleted but the numbers of cells slowly recover with time. By monitoring both cell type and CDw52 expression with FACS staining, this recovery can be followed. The T cells appear to be most severely affected and it is these cells that can exhibit loss of antigen. Publications now arising from several groups monitoring CHO Campath clinical trials, reveal that a number of the patients develop partially or fully CDw52 negative T cells (Hertenstein et al 1995, Brett et al 1996a). When studied at Wellcome Research Laboratories, totally antigen-negative T cells from patients were found to be also CD55 and CD59 negative (Rowan et al 1998). She demonstrated that the lack of antigen was not only a surface phenomenon but could also be visualised at the RNA level. Vanessa Taylor, another Ph.D student at Wellcome, utilised samples from partially GPI-defective patients to separate the CDw52 negative cells from positive cells by cell sorting. She found that the defect in the negative cells could be monitored by culturing the cells in radioactive precursors for GPI-anchor synthesis (Field and Menon 1992, Taylor et al 1997) but did not involve the glycotransferase gene PIG A. This work also determined that patients exhibited both cDNA sequences previously isolated from Wien 133 cells and

that both were detectable by Campath-1H antibody (Taylor et al 1997). This data could predict undesirable consequences for the use of some depleting antibodies in therapy. The reason for depleting CDw52 expressing cells in vitro is to remove graft versus host (GVH) reactive cells in bone marrow transplants or in vivo to either reduce the numbers of self reactive cells in autoimmune diseases or to ablate lymphocytic tumour cells. If in any of these situations, antigen negative variants arose, it could be a most undesirable consequence. Rawstron (et al 1999) have recently produced data demonstrating that some patients exhibit low numbers of CDw52 negative cells prior to immunotherapy and so it may be that after depletion with Campath-1H it is these cells that survive.

CHAPTER SIX: THE GENERAL DISCUSSION

This thesis was designed to clarify earlier observations that mononuclear effector cells isolated from volunteers responded differently in ADCC assays when targeted by either hamster CHO or rat YO Campath-1H (J.Tite, personal communication). Rademacher (et al 1988) and Leatherbarrow (et al 1985) had previously shown that the removal of antibody N-linked carbohydrate led to the loss of activity in certain cell based assays. As an extrapolation of this data plus our own observations, it had been hypothesised, that antibody isolated from different species of host cells might possess different N-linked sugars on the Fc CH2 domain. The project was planned to enable both the manufacture of Campath-1H antibody from mouse NSO cells, the media development for those cells and the comparison with CHO and YO Campath-1H in in vitro assays representing mechanisms thought to be utilised in vivo during immunotherapy. In addition oligosaccharides, released from the antibody isolates, were compared for carbohydrate composition.

The assays tabulated in Chapter Three were defined utilising Campath-1H sourced from CHO cells. Repeated ADCC and monocyte-mediated assays using effector cells purified from volunteers confirmed that responses did vary between donors but, the reasons why were never sought. Evidence from Moretta (et al 1993) regarding recognition of MHC class 1 type by NK cells and data produced from the classification of IgG Fc receptor polymorphism's (Koene et al 1997, Salmon et al 1992 and Sanders et al 1995) in relation to IgG binding may be relevant. To properly define the mechanisms involved in the assays it would be advantageous to characterise donors for both MHC 1 plus IgG Fc RII and RIII type prior to the experiment. It may even be possible to develop an immortalised NK effector cell line for use in ADCC assays. The removal of CD56 cells was always demonstrated to ablate the ADCC response and the Fc receptor blocking experiments implied that Fc RIII was the receptor mediating the effect. The observation that γ IFN could induce increased inhibition by monocytes was very exciting and could have implications for therapeutic use of antibodies such as Campath-1H in that lower concentrations of antibody might be required. This may reduce the side effects associated with Campath-1H administration. Unfortunately these were the last assays attempted and future work could centre around the investigation of cytokine involvement.

Assays studying the engagement of antigen on naturally expressing CDw52 cells or transfected cells were both reproducible and simpler to control. In these studies the presence of antibody carbohydrate was irrelevant and suggested that the mode of action was merely influenced by antigen recognition and not antibody conformation. The inclusion of unrelated antibodies in the assays substantiated the antigen specificity. There was obviously

a requirement for crosslinking as 80% aggregated CHO antibody alone could match crosslinked monomeric antibody whilst YO (22% aggregate) antibody could not. Perhaps the level of aggregation is important.

In Chapter Four the expression of Campath-1H antibody from mouse NSO cells is described and followed by the utilisation of the isolated antibody in the assays defined previously. The NSO Campath-1H antibodies tested were those isolated from cells during the process of media development, from serum-containing to protein-free media, as well as from cells cultured under fermentor conditions in the latter medium. The N-linked oligosaccharide content of the antibodies generated at each stage of development was isolated and investigated. Attempts to correlate these latter findings with the biological assay results were made and tabulated. For several antibody isolates (NSO.S, and .PF), the cells were cultured as monolayers on laboratory scale and the most obvious failing of this system was that the CO_2 content and pH of the media were not constantly monitored and could theoretically have led to differences in glycosylation (Patel et al 1992). The CHO and NSO fermentor batches of antibody were from cells which were maintained in suspension culture under defined conditions. The results presented compare favourably with the data of Lund (Lund et al 1993) who demonstrated that antibodies isolated from monolayers of cells, such as NSO.S and .PF, contained more galactose than cells cultured in suspension (NSO.F) and contradict Patel (Patel et al 1992) who showed that cells cultured in serum (NSO.S) contained lower levels of galactose than those cultured in serum-free medium (NSO.PF). The correlation of NSO carbohydrate content with assay result was more difficult to determine as the oligosaccharide was of three types altering in ratio and attempts to sequentially remove monosaccharides from CHO antibody had not been very successful. This work should certainly be repeated and the range of sugars to be removed extended. In terms of assays, NSO.S was the least active in ADCC but equivalent to other isolates in assays with no effectors. NSO.F antibody isolated from cells cultured under fermentor conditions proved to be similar to NSO.PF and .CF in ADCC but superior in monocyte assays. It could be suggested that, as levels of di-galactosylation decreased and agalactosylation increased, activity in ADCC improved. To fully define the relationship between assay response and carbohydrate composition it would be necessary to develop NSO cell lines deficient in characterised glycotransferases so that antibodies lacking in specific glycosylations could be generated. There are CHO cells of this nature (Lec series) but they are not dhfr deficient and so cannot be utilised for the CHO expression vectors described. It is theoretically possible to select for defined oligosaccharide structures with lectin columns but whether this is feasible for antibodies I am unsure. It is documented as being a difficult procedure due to the way in which the carbohydrate is buried within the CH2 domain (Wright and Morrison 1997). It may be easier to culture antibody producing

cells in the presence of various inhibitors which act at different steps in the glycosylation pathway prior to selection on lectin columns.

To overcome the problem of the incorrect glycosylation of therapeutic products it may be necessary for manufacturers to either utilise host cells deficient in the addition of all glycosylations and complete the process in vitro or develop transgenic host cell lines containing human glycostransferases. Recent experiments performed by Umana (Umana et al 1999) describe the use of CHO cells expressing chimeric anti-neuroblastoma IgG1 of low ADCC activity. These were transfected with the transferase gene β(1,4)-N-acetylglucosaminyltransferase lll. In the N-linked glycosylation pathway this gene, which is absent in murine and CHO cells, adds bisecting GlcNAc and by controlling the level of gene expression with drug selection the level of bisecting GlcNAc addition could be modified. It was found that as antibody bisecting GlcNAc content increased, ADCC activity improved substantially. This supports the hypothesis of this thesis and re-enforces the finding that YO derived Campath-1H antibody, which contains bisecting GlcNAc (Lifely et al 1995), was superior to both CHO and NSO derived antibody in ADCC assays. Naturally occurring bisected oligosaccharides form 18% of human IgG (Furakawa and Kobata 1991).

Some of the most interesting data obtained was that from NSO.IgG4 antibody because whilst acting as expected in ADCC assays i.e. not inducing target cell lysis, it was found to be as active as NSO.IgG1 in both crosslinking and monocyte assays. Even deglycosylation of NSO.IgG1 and NSO.IgG4 failed to ablate their activity in these assays. This is similar to data published reporting that aglycosylated IgG3 can mediate the phagocytic respiratory burst (Pound et al 1993a). It would have completed this aspect of the project if experiments designed to identify the receptors involved in the monocyte assays had been more informative or if deglycosylated antibody had been assessed for receptor usage. Data not presented implied that receptors Rl and Rll were contributing to the inhibitive effect but the assays were unreliable especially as far as demonstrating the involvement of Fc Rlll. These assays should be properly controlled and repeated. It would be interesting to determine whether IgG4 interacts with Fc Rlll or not.

The final results chapter, Chapter Five, encompasses the data from the isolation and cloning of two similar CDw52 open reading frame cDNA sequences which had been isolated from Wien 133 cells. The cDNA open reading frames differed at two nucleic acids which led to two altered amino acids. Interestingly, the amino acids were not within the region which formed the mature Campath antigen but, were in the portion of cDNA governing GPI-anchorage (Moran and Caras 1991b). When the cDNA, which is accepted to be that of CDw52 (Xia et al 1991), was cloned into vectors and expressed in both CHO cells and Jurkat T cells it led to detectable surface expression. The alternative cDNA did not lead to

expression in CHO cells but was later found to be expressed competently in Jurkat cells. The divergence in Campath-1H detectable expression in CHO cells was thought to be caused by the loss of CDw52 epitope as a result of aberrant GPI-anchorage. This was investigated via the construction of CD4/CDw52 chimeric cDNA which utilised the extracellular domain of the antigen CD4 linked to either of the CDw52 GPI-anchor determining cDNA sequences. On expression in CHO cells, both chimeras led to detectable CD4 surface expression which differed from the conventional CD4 in molecular weight and PIPLC sensitivity. Both chimeras were of lower molecular weight, indicating the loss of some of their transmembrane and cytoplasmic domains plus they were both PIPLC sensitive which demonstrated the presence of GPI-anchors. It was only at this point that both CDw52 cDNAs were introduced into Jurkat cells and surface CDw52 expression demonstrated for each. It indicates that CHO cells may lack certain glycotransferases. The studies on the Wien cell cDNA coincided with the findings of CDw52 negative patients arising from Campath-1H therapy (Brett et al 1996a, Hertenstein et al 1995) and was initially thought to reflect the in vivo data. This was not to be but, patients were demonstrated to possess one or other or both forms of CDw52 cDNA (Taylor et al 1997) and the defect shown to involve GPI-anchor synthesis (Hertenstein et al 1995, Taylor et al 1997). Derivation of the Campath negative cell lines MR4 and CV4 (Rowan et al 1998) proved to be advantageous as their use in ADCC assays revealed the possibility of those cells evading immune intervention in vivo. Since that time it has been demonstrated that some patients have a low percentage of CDw52 negative cells prior to treatment which may be selected for by Campath therapy (Rawstron et al 1999).To finalise this aspect of the thesis it would be necessary to determine the exact defect, if any, in the CDw52 negative cells by checking genes which are involved in the early stages of GPI-anchor biosynthesis.

The data presented here does appear to support the original hypothesis that method of culture and culture medium are able to influence antibody functional ability. Extension of the project would strengthen the case.

CHAPTER SEVEN: REFERENCES

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The use of serum-free medium for the production of functionally active humanised monoclonal antibody from NS0 mouse myeloma cells engineered using glutamine synthetase as a selectable marker

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Abstract

A protein-free growth medium (W38 medium) had previously been developed for the NS0 mouse myeloma cell line which is cholesterol-auxotrophic. This paper describes the development of a protein-free growth medium for NS0 cells expressing humanised monoclonal antibody using GS (glutamine synthetase) as a selectable marker. Several GS-engineered NS0 cell lines expressing humanised monoclonal antibody grew in a modification of W38 medium which maintained GS-selection, supplemented with cholesterol, phosphatidylcholine and β-cyclodextrin. Further studies showed that additional glutamic acid, asparagine, ribonucleosides and choline chloride improved cell growth. Amino acid analysis identified a number of amino acids that were being depleted from the culture medium. NS0 cell lines 9D4 and 2H5 expressing CAMPATH-1H* were adapted to enable them to grow serum-free in the absence of cholesterol and β-cyclodextrin. Cholesterol-independent 9D4 (9D4.CF) cells grown in shake flask culture using an enriched protein-free medium (WNSD medium), supplemented with human recombinant insulin (Nucellin), reached a maximum cell density to 1.86×10^6 cells ml^{-1} producing 76.6 mg l^{-1} of antibody. CAMPATH-1H antibody produced using serum-free medium was found to be functionally active *in vitro* in the Antibody Dependant Cellular Cytotoxicity (ADCC) assay.

Abbreviations: C – cholesterol; CD – cyclodextrin; dhfr – dihydrofolate reductase; F68 – Pluronic F68; GS – glutamine synthetase; MSX – methionine sulphoximine; P – phosphatidylcholine; PC-FBS – phosphatidylcholine, cholesterol and foetal bovine serum; RPMI – RPMI 1640 medium, ADCC – Antibody-dependant cellular cytotoxicity

Introduction

NS0 (Galfre and Milstein, 1982) is a cholesterol-auxotrophic (Keen and Steward, 1995) mouse myeloma cell line that does not contain endogenous glutamine synthetase (GS) (Brown *et al.*, 1992) and is therefore unable to synthesise glutamine from other amino acids. For this reason NS0 cells have an essential requirement for exogenous glutamine and are particularly well suited for use in recombinant gene technology which utilises glutamine synthetase as a selectable marker (Bebbington *et al.*, 1992). The GS expression system exploits the lack of cellular GS in the host cell line using a plasmid containing both the GS gene and the product gene. The plasmid contains the DNA encoding for the antibody heavy and light chains driven by the strong hCMV promoter regions and DNA encoding for glutamine synthetase controlled by the weak SV40 promoter. Transfected cells can be isolated by culturing in a selection medium which lacks glutamine. The GS and product gene can be co-amplified

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by exposure with methionine-sulphoximine (MSX), a GS-inhibitor.

Monoclonal antibodies from different mammalian species can be humanised by grafting the complementary determining regions (CDRs) from the antibody of one species on to a suitable human antibody donor framework (Winter and Milstein, 1991). Humanised antibodies are expected to be of greater therapeutic use in man than chimeric or murine antibodies which are known to generate an anti-species or an anti-frame work immune response, such as the human anti-mouse antibody response (HAMA response) (Wallace *et al.*, 1994; Isaacs, 1990). An additional advantage of humanisation is that the isotype of the human Fc framework can be selected to support the desired effector functions.

Cloned GS-engineered NS0 cell lines expressing CAMPATH-1H or humanised anti-CD2 monoclonal antibody were used in this study. CAMPATH-1H is a humanised IgG1 monoclonal antibody (Riechmann *et al.*, 1988) which binds to the CD52 antigen, which is an abundant glycoprotein present on the vast majority of human lymphocytes (Hale *et al.*, 1990), monocytes and most lymphoid malignancies (Hale *et al.*, 1988b). CAMPATH-1 antibodies have been used successfully in the therapy of transplant rejection (Hale *et al.*, 1988a), rheumatoid arthritis (Isaacs *et al.*, 1992) and non-Hodgkins lymphoma (Hale *et al.*, 1988b). Previously, CAMPATH-1H had been engineered into the CHO DUK B11 cell line (Urlaub and Chasin, 1980) using a dihydrofolate reductase (dhfr) selection system (Page and Sydenham, 1991). CAMPATH-1H is known to initiate cell death through antibody-dependant cellular cytotoxicity (ADCC) (Hale *et al.*, 1985; Dyer *et al.*, 1989).

Fully defined protein-free media offers a number of potential advantages for manufacture of antibodies, vaccines and other therapeutic products from mammalian cells relating to cost, reproducibility, regulatory considerations and purification of product. Protein-free culture media are now available that will support the growth of some hybridoma and myeloma cell lines. Cells that require exogenous cholesterol, such as NS0, will not grow in these media. We have described the growth of NS0 cells in W38 protein-free medium containing cholesterol rich lipid with β-cyclodextrin and their subsequent adaptation to cholesterol-independence (Keen and Steward, 1995). In this study a protein-free medium was developed for GS-engineered NS0 cells which supported cell growth and the production of functionally active antibody.

Materials and methods

Media and reagents

The following materials were used: Glutamine, non-essential amino acids, and trypan blue were obtained from ICN-Flow (High Wycombe, UK); RPMI 1640 product 31870–025 (RPMI), Dulbecco's medium product 21969–035 (DMEM), DMEM without ferric nitrate product 73-860–54 and Iscove's modified Dulbecco's medium (Iscove's) product 21980–032 from Life Technologies (Paisley, UK); dialysed and non-dialysed New Zealand foetal bovine serum (FBS) from Bioclear (UK); ferric citrate product 28381 from BDH (Poole, UK); tissue culture plastic from Costar (Badhoevedorp, Holland) and Erlenmeyer flasks from Corning (New York, USA). The cyclodextrins used were a generous gift from Wacker Chemicals (Munich, Germany). All other Chemicals and supplements were obtained from Sigma (Poole, UK).

Ferric citrate was dissolved at 10 mg ml^{-1} in boiling water, allowed to cool, then 0.2 μm filtered using a Millex GV filter (Millipore, France). Cyclodextrins were prepared as a 10 mg ml^{-1} solution in water then 0.2 μm filtered. WNSA, WNSB and WNSD media (Table 2) were prepared by the Medium Production Unit, Wellcome (method of preparation available on request).

Preparation of vesicles containing phosphatidylcholine and cholesterol

Phosphatidylcholine (P) and cholesterol (C) were dissolved at 10 mg ml^{-1} in absolute ethanol. A cholesterol rich lipid solution (CR-lipids) was prepared by mixing P and C in a ratio of 2:1 with 0.25 mg ml^{-1} α-tocopherol to give a final total concentration of 10.25 mg ml^{-1}. For experimental purposes this solution was treated as being sterile. Both cholesterol and phosphatidylcholine have a very low solubility in aqueous solution; cholesterol forms a precipitate while phosphatidylcholine becomes uniformly suspended in solution. When phosphatidylcholine and cholesterol are mixed together in ethanol, then rapidly added to aqueous solution small unilamellar vesicles are formed (Stryer, 1988). Vesicles were thus prepared in the culture medium by the addition of CR-lipids to give a final concentration of 10.25 mg l^{-1}.

GS-engineered cell lines and routine culture conditions

The serum-containing GS-selection medium used ('select' medium) was a modification of Celltech 'GS-selection medium' (Bebbington and Yarrington, 1989) which contained DMEM without ferric nitrate with 60 mg l^{-1} glutamic acid (G) and asparagine (A), 7 mg l^{-1} adenosine, guanosine, cytidine and uridine, 2 mg l^{-1} thymidine, ICN-Flow non-essential amino acids at single strength and 5% dialysed FBS. Only cells which express GS are able grow in this medum.

In order to express antibody in NS0 cells, the antibody heavy and light chain cDNA's were cloned into glutamine synthetase selection based vectors obtained from Celltech Ltd, Slough, UK. This construct was transfected into the NS0 cell line by electroporating twice using a Bio Rad Gene Pulser set at 1500 volts and 3 μF. The transfected cells were cultured in using GS 'select' medium. Screening by ELISA assay identified the colonies that produced the highest level of antibody. High secreting single colonies were expanded, amplified by exposure to various concentration (5–50 μm) of MSX, then dilution cloned.

NS0 9D4.5A11 (9D4) and 9D4.4F8 (4F8) expressing CAMPATH 1H were derived from the same primary well and amplified with 5 μM MSX. NS0 2H5 (2H5) expressing CAMPATH 1H was amplified with 10 μM MSX. Specific antibody production rates (SPR) for 9D4, 4F8 and 2H5 were 11, 17 and 4 μg 10^6 cells^{-1} day^{-1}. The SPR was determined over 24 hours, in a sub-confluent static culture using serum-containing 'select' medium. The 8C9.50B5 (8C9) cell line which expressed humanised anti-CD2 antibody was not amplified with MSX. These cells were routinely grown in static culture using 75 cm^2 flasks containing 30 ml of serum-containing 'select' medium with MSX which was incubated at 37 °C with 7.5% CO_2. The cells grew slowly, lightly attached to the plastic reaching a maximal concentration of around 6×10^5 cells ml^{-1}. Every few days the cells were diluted to approximately 10^5 cells ml^{-1} in fresh growth medium . The adherent cells were easily removed from the plastic by firmly tapping the side of the flask. Non-engineered NS0 cells were grown in static culture using DMEM, 5% FBS and 2 mM l-glutamine (NS0 growth medium).

Growth curves were performed using duplicate shake flask cultures. 250 ml Erlenmeyer flasks containing 35 ml of culture were incubated at 37 °C in an Innova shaking incubator (New Brunswick Scientific, USA) and shaken at 100 revolutions per minute (rpm). The cultures were re-gassed daily for half a minute with 5% CO_2 and a sample removed for cell counting and antibody assay. Daily gassing ensured that cell growth was not inhibited by O_2 depletion or CO_2 build up.

Washing procedure and cell counting method

Cells grown in serum-containing medium were washed by pelleting at 1200 × g for 5 minutes at +4 °C, resuspended in protein-free medium without addition, pelleted again, resuspended in fresh medium at 2×10^6 cells ml^{-1}, then diluted to the required concentration in the media under test. All media were pre-warmed to room temperature before use.

Culture samples were diluted with an equal volume of 0.1% w/v isotonic trypan blue (trypan blue) , mixed by pipetting, then counted using trypan blue exclusion. In serum-containing static cultures the cells were first dislodged by tapping the side of the flask.

Amino acid analysis

Amino acid composition of the culture medium was analysed before and after cell growth in order to determine usage by the cells. Centrifuged medium samples were first hydrolysed by addition of HCl to a final concentration of 0.1 M and proteins removed by precipitation with 80% ethanol. The supernatant was dried (speedvac concentrator), reconstituted with a 250 ppm tripotassium EDTA solution and passed through a filter with a 10,000 molecular weight cut off. The amino acid composition was then determined using an Applied Biosystems model 420A dedicated derivitiser/amino acid analyser.

Detection of antibody levels by ELISA and functional activity by ADCC

Culture supernatant was clarified by microfuge centrifugation at 13 000 rpm and stored at +4 °C for up to 2 weeks prior to assay. The amount of humanised antibody was determined using a sandwich ELISA assay. The wells of a 96 well maxisorp ELISA plate (Nunc, Roskilde, Denmark) were coated with 2 μg ml^{-1} sheep anti-human IgG heavy and light chain (Seralab, UK, cat. no. SDL2015) in phosphate buffered saline (PBS). The excess protein binding sites were blocked with diluent (1% Bovine serum albumin fraction V in PBS). The binding of triplicate antibody samples to the coated

wells was detected using peroxidase conjugated sheep anti-human IgG and then measured by the colour generated from the tetra-methyl benzidine substrate (Sigma, Poole, UK). The reaction was stopped with 2 M H_2SO_4 and the colour measured at an absorbance of 450 nm. The amount of antibody in the test samples was quantified from a curve generated using a purified CAMPATH-1H antibody standard. Between each step of the ELISA the plates were washed 6 times with wash buffer (PBS with 0.05% Tween 20 detergent).

For the Antibody dependant cellular cytotoxicity assay (ADCC) peripheral blood mononuclear cells (PBMC) were separated from defibrinated fresh human blood by centrifugation over a lymphoprep (Nycomed) gradient. Wien 133, a human B cell lymphoma cell line (Nacheva *et al.*, 1987) expressing the CD52 antigen on its cell surface membrane was grown in log phase, labelled with ^{51}Cr, washed, resuspended at 2×10^5 ml^{-1}, then aliquoted into 96 well 'U' bottomed tissue culture plates at 50 μl well^{-1}. Wien 133 were cultured using Iscove's medium, 10% FBS and 2 mM l-glutamine (Wien growth medium). CAMPATH-1H antibody dilutions (50 μl) in Wien growth medium, were added to triplicate wells in the plate. Nine control wells without antibody were also included in each assay. The plates were incubated at 37 °C for 1.5 hours in a humid CO_2 incubator prior to the addition of 100 μl of PBMC effector cells at a concentration of 2.5×10^6 ml^{-1}, in Wien growth medium, to each antibody-containing well. This resulted in a ratio of 25 effector cells for each target cell. The effector cells were also added to one triplicate set of control wells (non-antibody mediated lysis control), Wien growth medium alone was added to yet another set of control triplicates (spontaneous lysis control) and 100 μl of 1% w/v Triton X100 detergent in H_2O was added to the final set of control wells (total release control). The plates were spun for 5 minutes at 1500 rpm before being replaced at 37 °C for 5 hours. At the end of the incubation period 100 μl of medium was removed from each well and counted in a γ counter (Wallac Wizard, model 1470, Turku, Finland). The level of specific release was calculated as follows:

$$\%\,\text{specific release} = \frac{(\text{mean cpm. antibody mediated release}) - (\text{mean cpm. spontaneous release}) \times 100}{(\text{mean cpm. total release}) - (\text{mean cpm. spontaneous release})}$$

Results

Growth characteristics in serum-containing 'select' medium

Each of the GS-engineered NS0 cell lines grew differently in serum-containing 'select' medium. Duplicate static cultures of line 9D4 seeded at 10^5 cell ml^{-1} reached an average density of 5.75×10^5 cells ml^{-1}, while line 8C9 reached an average density of 12.9×10^5 cells ml^{-1}. NS0 9D4 cells were pre-adapted to suspension growth in 'select' medium. Duplicate shake flask cultures of these cells, seeded at 10^5 cells ml^{-1}, reached an average density of 6.75×10^5 cells ml^{-1} after 4 days with an average antibody level 12.01 mg l^{-1} after 6 days.

The protein-free growth of GS engineered NS0 cells

GS-engineered cells require glutamine-free culture medium in order to maintain selection pressure. Glutamine synthetase produces glutamine directly from glutamic acid and indirectly from other amino acids such as asparagine. The catabolism of glutamine is essential for the *de novo* synthesis of ribonucleosides. GS-selection media should therefore contain glutamic acid, asparagine and ribonucleosides but be free of glutamine. W38 protein-free medium (Keen, 1995) supplemented with CR-lipids and β-cyclodextrin supports high density growth of non-engineered NS0 cells. A modification of W38 medium, designated WNSA, was produced (Table 1), which was free of glutamine and contained additional glutamic acid, asparagine, adenosine, guanosine, cytidine, uridine and thymidine.

9D4 and 2H5 cells were adapted to protein-free growth in 25 cm^2 flasks. Cells grown in serum-containing 'select' medium were washed using WNSA medium, resuspended at 10^5 cells ml^{-1} in 5 ml WNSA medium supplemented with CR-lipids and cyclodextrin (WNSA + lipids), then incubated at 37 °C with 7.5% CO_2. Every few days the cells were subcultured by dilution with fresh medium taking great care to ensure that the cells did not overgrow. Both 9D4 and 2H5 readily adapted to protein-free growth (Tables 2A and 2B), with the non-viability remaining between 10% to 30% throughout. The high non-viability may have been due to cell death occurring immediately after

Table 1. Composition of WNSA and WNSD select medium. The final osmolality of these media was between 330 and 350 mOSM. The differences in WNSD medium are highlighted

	Final concentration of medium components mg l^{-1}				
	WNSA	WNSD		WNSA	WNSD
Amino acids			**OTHER COMPONENTS**		
L-Alanine	0	0			
L-Arginine	142	**192**	EDTA[a]	6	6
L-Asparginine	85	**505**	Oxalacetic acid	2	2
L-Aspartic acid	10	**20**	Progesterone	0.006	0.006
L-Cystine	61.7	61.7	Sodium nitroprusside	5	5
L-Glutamic acid	70	**490**	Taurine	30	30
Glycine	20	**30**	Tween-80	0.2	0.2
L-Histidine	28.5	**58.5**	Glucose	6250	**3000**
L-Hydroxy-proline	10	10	Hypoxanthine	0.272	0.272
L-Isoleucine	77.4	**177.4**	Adenosine	7	**14**
L-Leucine	77.4	**177.4**	Guanosine	7	**14**
L-Lysine	93	**193**	Cytidine	7	**14**
L-Methionine	22.5	**82.5**	Uridine	7	**14**
L-Phenylalanine	40.5	**60.5**	Phenol red	10	10
L-Proline	10	10	Putrescine	2.2	2.2
L-Serine	36	36	Pyruvic acid	165	165
L-Threonine	57.8	57.8	Thymidine	2.41	**4.81**
L-Tryptophan	10.5	**30.5**	MOPS buffer	3000	3000
L-Tyrosine	57.2	57.2	Sodium bicarbonate	2850	2850
L-Valine	56.8	**116.8**			
			TRACE ELEMENTS		
VITAMINS			Aluminium chloride	0.001	0.001
δ-Pantothenic acid	2.1	2.1	Barium chloride	0.002	0.002
Choline chloride	4.2	**104.2**	Calcium chloride	167	167
Flavin adenine-dinucleotide	0.02	0.02	Chromium potassium-sulphate	0.001	0.001
Folic acid	2.5	2.5	Copper sulphate	0.0025	0.0025
Glutathione	0.5	0.5	Ferric citrate	1	1
i-Inositol	21	21	Ferrous sulphate	0.8	0.8
Nicotinamide	2.5	2.5	Lithium chloride	10	10
P Aminobenzoic acid	0.5	0.5	Magnesium sulphate	150	150
Pyridoxin	2.5	2.5	Molybdic acid	0.0001	0.0001
Riboflavin	0.3	0.5	Nickel chloride	0.0002	0.0002
Thiamin	2.5	2.5	Potassium bromide	0.0001	0.0001
α-tocopherol	0.00025	0.00025	Potassium chloride	400	400
Cyanocobalamin	0.68	0.68	Potassium iodide	0.00001	0.00001
Biotin	0.1	0.1	Rubidium chloride	0.00001	0.00001
			Silver chloride	0.000005	0.000005
OTHER COMPONENTS			Sodium chloride	6200	6200
Adenine	0.4	0.4	Sodium fluoride	0.004	0.004
Citric acid	25	25	Sodium phosphate	471	471
Phosphatidylcholine	1	1	Sodium selenite	0.03	0.03
Ethanolamine	3	3	Stannous chloride	0.0001	0.0001
			Zinc sulphate	0.8	0.8

[a] EDTA = Ethylenediaminetetraacetic acid-disodium salt.

Table 2. Adaptation to serum-free growth

Pass number	day	viable	non-viable	subcultured	% residual FBS
		Cell count × 10^{-5} cells^{-1}			
Table 2A, line 9D4					
1	7	5.3	4.6	1 to 8	0
2	13	2.0	0.65	1 to 4	0
3	18	2.45	0.6	1 to 3	0
4	20	2.4	0.25	1 to 3	0
5	25	3.0	0.65	1 to 5	0
6	30	3.0	0.8	cryopreserved	
Table 2B, line 2H5					
1	7	7.15	0.5	1 to 8	0
2	13	2.3	0.7	1 to 4	0
3	18	6	0.95	1 to 3	0
4	20	3.8	0.65	1 to 3	0
5	25	4.4	0.95	1 to 5	0
6	30	3.5	0.9	cryopreserved	
Table 2C, line 4F8					
1	5	5.0	2.95	1 to 5	0.2
2	10	9.1	2.7	1 to 5	0.04
3	14	4.2	1.1	1 to 5	0.008
4	19	3.7	1.3	1 to 5	0.016
5	24	10.9	3.2	1 to 5	0 (cells washed)
6	28	5.4	0.7	cryopreserved	

Tables 2A and 2B. The NS0 **9D4** and **2H5.10** cell line secreting CAMPATH-1H antibody **were washed to remove serum** then adapted to growth in **WNSA** protein-free medium containing Nucellin, β-cyclodextrin and cholesterol-containing lipids.
Table 2C. The NS0 **4F8** cell line secreting CAMPATH-1H antibody was adapted to growth in **WNSB** protein-free medium containing recombinant insulin, β-cyclodextrin and cholesterol-containing lipids. **The residual FBS was diluted on sequential passage** then finally remove by washing the cells after 24 days in culture.

subculture and to the relatively slow growth rate.

An attempt was made to improve adaptation by using a weaning process. Cells in serum-containing medium were diluted in WNSA + lipids without washing and the residual serum sequentially diluted out as the cells were passaged. This method of adaptation (Table 2C) was also successful but seemed to offer no benefit over direct transfer to protein-free growth. Out of 11 different GS-engineered cell lines that were transferred to grow in WNSA + lipids, 10 adapted to protein-free growth. The other line grew only in WNSA + lipids containing glutamine suggesting that it was unable to synthesise glutamine when cultured in this medium. 9D4 , 2H5 and 8C9 were sequentially passaged in WNSA + lipids. The addition of 5 mg l^{-1} of human recombinant insulin (Nucellin) slightly increased the cell growth rate and improved viability (data not shown). For this reason Nucellin was then routinely added to the medium.

Enrichment of WNSA protein-free medium with additional glutamic acid, asparagine and ribonucleosides (select additives) improves growth and antibody secretion

WNSA medium was enriched to try and improve cell growth. Figure 1 shows the growth of serum-free adapted 9D4, 2H5 and 8C9 in 5 ml static cultures with WNSA medium containing additional select additives. In the cultures with 'extra ribonucleosides' or 'extra

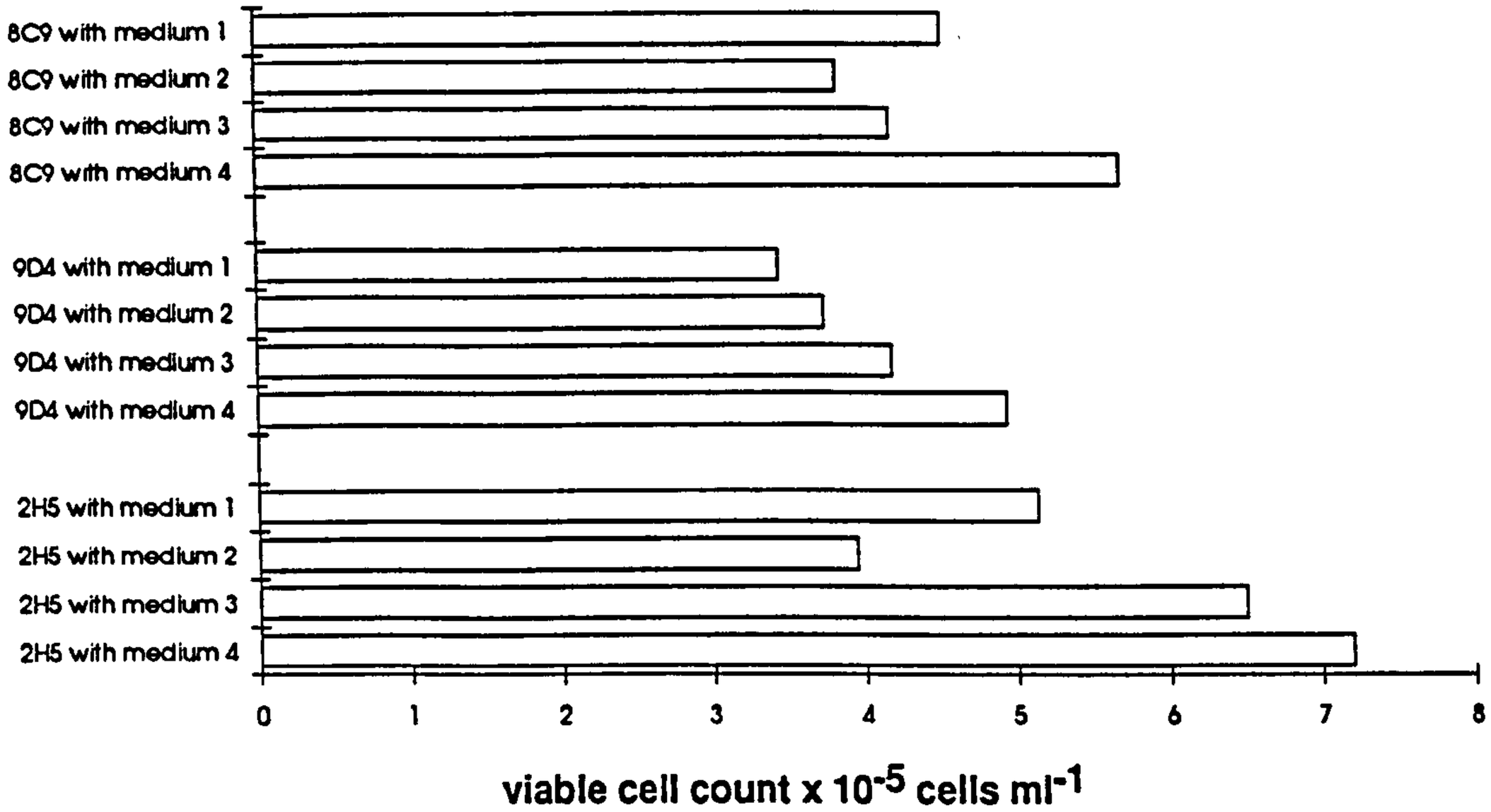

Fig. 1. Effect of adding extra amino acids and ribonucleosides on the growth of three GS-engineered NS0 cell lines in medium 1. WNSA protein-free medium with lipid, β-cyclodextrin and recombinant insulin (WNSA + lipids), medium 2. WNSA + lipids with extra ribonucleosides, medium 3. WNSA + lipids with extra G+A and medium 4. WNSA + lipids with extra G+A and ribonucleosides. Mean counts of duplicate shake flask cultures. G + A = glutamic acid and asparagine.

G+A' the level of ribonucleosides was doubled, glutamic acid was at 190 mg l^{-1} and asparagine at 205 mg l^{-1}. Duplicate 5 ml cultures seeded at 0.5×10^5 cells ml^{-1} in 25 cm^2 flasks were incubated with 7.5% CO_2. For all three cell lines, best growth occurred in the presence of additional G+A and additional ribonucleoside ('fully supplemented' medium). The cell concentration in the 'fully supplemented' 9D4, 2H5 and 8C9 cultures were respectively 4.95, 7.2 and 5.7×10^{-5} cells ml^{-1}, with antibody concentrations of 29.1, 13.8 and 8 mg l^{-1}.

Amino acid and glucose utilisation of GS-engineered NS0 in WNSA medium

In order to examine nutrient depletion, media samples pooled from duplicate cultures of 9D4, 2H5 or 8C9, in 'fully supplemented' WNSA medium were harvested after 6 days growth and the amino acid, glucose and lactate composition analysed (Table 3). The nutrient levels detected after 6 days growth were compared with the levels detected in unused culture medium. Several amino acids notably glutamic acid, asparagine, histidine, valine, methionine, cystine, isoleucine and leucine were heavily depleted with both glutamic acid and leucine being practically exhausted. In contrast glucose levels in the medium did not appear to be growth limiting.

Adaptation of NS0 9D4 to cholesterol independence

One aim of this study was to achieve cell growth in a medium free from animal derived components. Cholesterol is the only animal derived component in WNSD medium. However, NS0 cells have an essential requirement for cholesterol, which is extracted from Sheep wool. The use of β-cyclodextrin and lipid vesicles to supply the cholesterol requirement of NS0 cells caused several problems. Addition of cholesterol-containing vesicles to the medium resulted in lipid precipitation and increased cell death (data not shown). The shelf life of media containing vesicles was limited as after a few days +4 °C storage a lipid precipitate slowly formed. A precipitate could also be seen in slow growing cultures, after several days incubation, resulting in reduced cell viability. The precipitation problem could be reduced by the use of cholesterol:methyl-β-cyclodextrin complex (Sigma product C3175).

It was decided to adapt the 2H5 and 9D4 cell lines to cholesterol-independence using a method similar to that used for non-engineered NS0 cells (Keen and Steward 1995). Briefly, 0.1 ml of CR-lipids at a total

Table 3. Amino acid (aa) utilisation by the GS-engineered NS0 cell lines

	Actual amino acid of content of unused medium	Amino acid levels detected by the analyser in			
		unused medium	8G9 supn't	2H5.10 supn't	9D4 supn't
L-aspartic acid	10	26.3	11.3	10.3	13.3
L-glutamic acid	190	170.7	0	0	0
L-asparagine	205	205.8	35.6	4.6	136
L-serine	36	44.6	24.7	41	33.9
L-glutamine	0	6.9	4.4	8.8	9.9
L-glycine	20	21	15.4	15	16.1
L-histidine	28.5	21.3	8.1	12	9.3
L-taurine	30	23.4	21.9	23.4	22.5
L-arginine	142	121.4	78.7	90.9	49.2
L-threonine	57.8	61.3	35.7	44.6	35.1
L-alanine	0	3.1	37.8	81.7	44.1
L-proline	10	9.2	5.8	21	4
L-tyrosine	57.2	44.3	24.9	29	28.5
L-valine	56.8	54.7	9.7	14	12.9
L-methionine	22.5	24.2	5.6	6	6.7
L-cystine, L-cysteine	61.7	39.0	15.0	14.4	14.4
L-isoleucine	77.4	76	9.2	10.5	5.2
L-leucine	77.4	74.3	1.6	4.3	0
L-phenylalanine	40.5	49.1	24.3	21.9	17.7
ornithine	0	0	6.8	9.7	40.1
L-tryptophan	10.5	3.6	4.6	4.1	2.6
L-lysine	93	79.9	32.1	40.5	35.8
glucose	6250	6164	ND	ND	4490
lactate	0	0	ND	ND	1350

The first column shows the actual level of each amino acid in unused culture medium. The other four columns show the levels detected in unused medium and in culture medium harvested from cultures of NS0 8G9, 2H5.10 and 9D4 cells grown in static culture for 6 days using 'supplemented' WNSA medium. The highlighted amino acid values are much lower than in the unused culture medium, indicating that they were being depleted from the medium.

lipid concentration of 10.25 mg ml^{-1} in ethanol was added to 10 ml of dialysed FBS (CR-FBS). Cells growing in WNSA + lipid medium were subcultured into WNSA medium containing 1% CR-FBS, but lacking CR-lipids or β-cyclodextrin. The 9D4 cells were subsequently subcultured four times with 0.3% CR.FBS, then cultured serum-free and cholesterol-free, whereas the 2H5 cells were sequentially subcultured once with 1% CR.FBS, twice with 0.5%, twice in 0.15%, then grown cholesterol-free. The adapted cell lines were renamed 9D4.CF and 2H5.CF respectively.

Enriched WNSA medium with additional amino acid, ribonucleosides and choline gave increased biomass and antibody secretion

The effect of further medium enrichment on cell growth was investigated using potentially beneficial additives identified in the nutrient depletion studies. A modification of WNSA protein-free medium (WNSB) was prepared in which the glucose level was reduced to 3 g l^{-1}, asparagine increased to 265 mg l^{-1} and glutamic acid increased to 250 mg^{-1}. A 100× concentrate amino acid supplement (GS-aa) was prepared in water containing 10 mg ml^{-1} isoleucine, leucine and lysine, 6 mg ml^{-1} valine and methionine, 5 mg ml^{-1} arginine, 3 mg ml^{-1} histidine, 2 mg ml^{-1} phenylalanine and tryptophan with 1 mg ml^{-1} glycine and

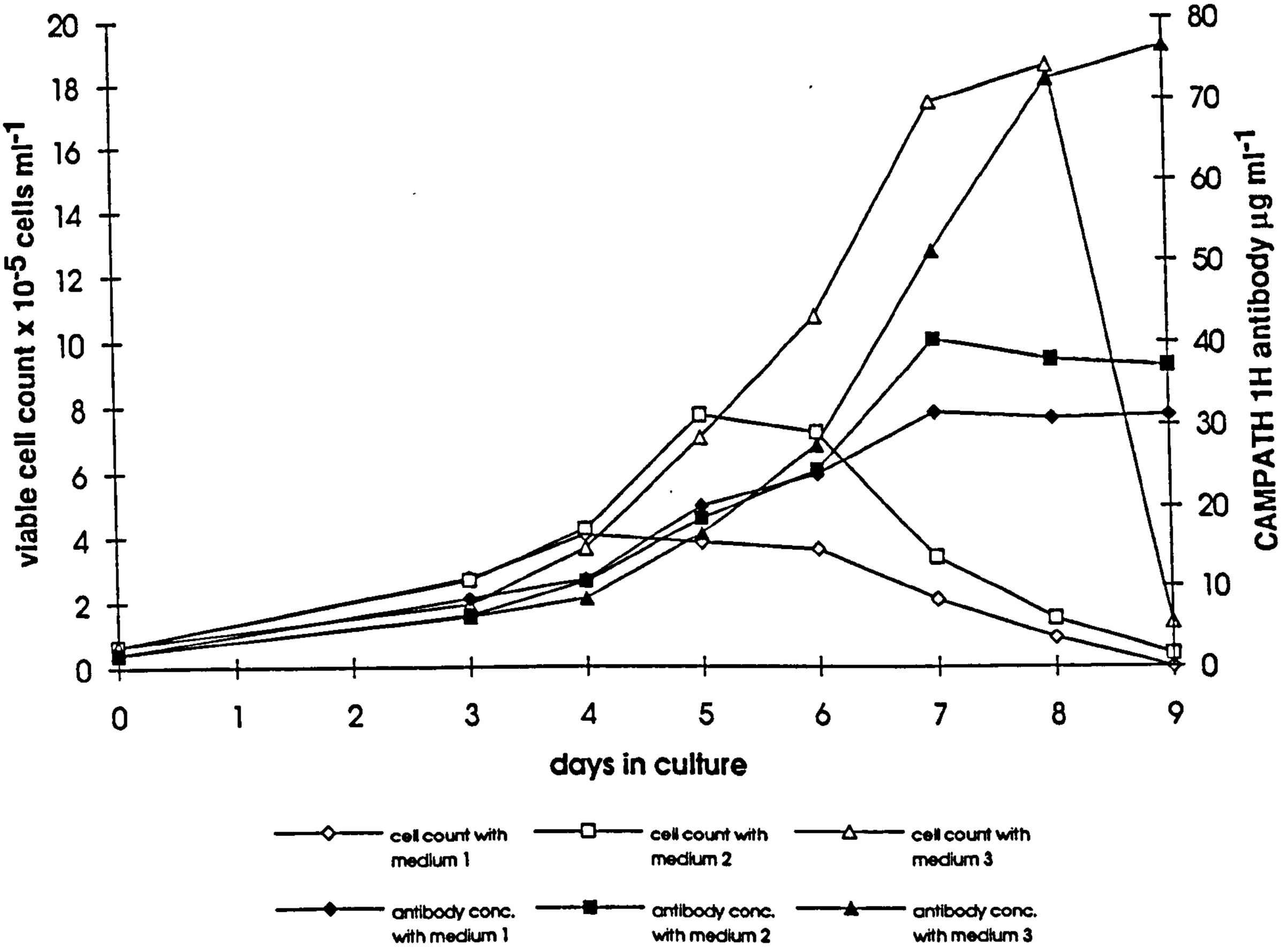

Fig. 2. NS0 9D4.CF cell growth and antibody production in medium 1. WNSB medium with Nucellin, medium 2. WNSB medium with Nucellin, GS-aa, extra ribonucleosides and extra G + A or medium 3. WNSB medium with Nucellin, GS-aa, extra ribonucleosides, extra G + A and choline chloride. Mean of duplicate shake flask cultures. G + A = glutamic acid and asparagine.

aspartic acid. In previous unpublished studies it was found that choline improved Y0 mouse myeloma and Chinese hamster ovary cell growth. A 100× concentrate solution containing 100 mg ml^{-1} choline chloride was prepared in water. These solutions were sterilised by filtration.

Figure 2 compares the growth of 9D4.CF cells in WNSB medium with Nucellin supplemented with additional nutrients using duplicate shake flasks seeded with 10^5 cells ml^{-1}. Medium 2 and 3 contained double ribonucleosides, 490 mg l^{-1} glutamic acid and 505 mg l^{-1} asparagine. The choline chloride in medium 3 had a dramatic effect on growth increasing the maximal cell density to 18.55×10^5 cells ml^{-1} with 76.6 mg l^{-1} antibody. The composition of medium 3 (without Nucellin) was renamed WNSD (Table 1). In WNSD medium with added Nucellin the cell density and accumulated antibody was far higher than in serum-containing select medium. Amino acid analysis of supernatant from the 7 days culture with WNSD with Nucellin (Figure 3) showed that there was a high rate of depletion of asparagine, glutamic acid, isoleucine and leucine. From this data it seemed probable that a lack of glutamic acid, isoleucine and leucine was inhibiting cell growth. Unfortunately, increasing the amino acid levels resulted in toxicity and inhibited cell growth. Removal of Nucellin had no detectable effect on cell growth or antibody production (data not shown). Electronmicrographs of cells in late log phase showed a condensation of chromatin that is characteristic of death by apoptosis. Further development of this medium for use in fed batch culture in stirred bioreactors will be reported elsewhere.

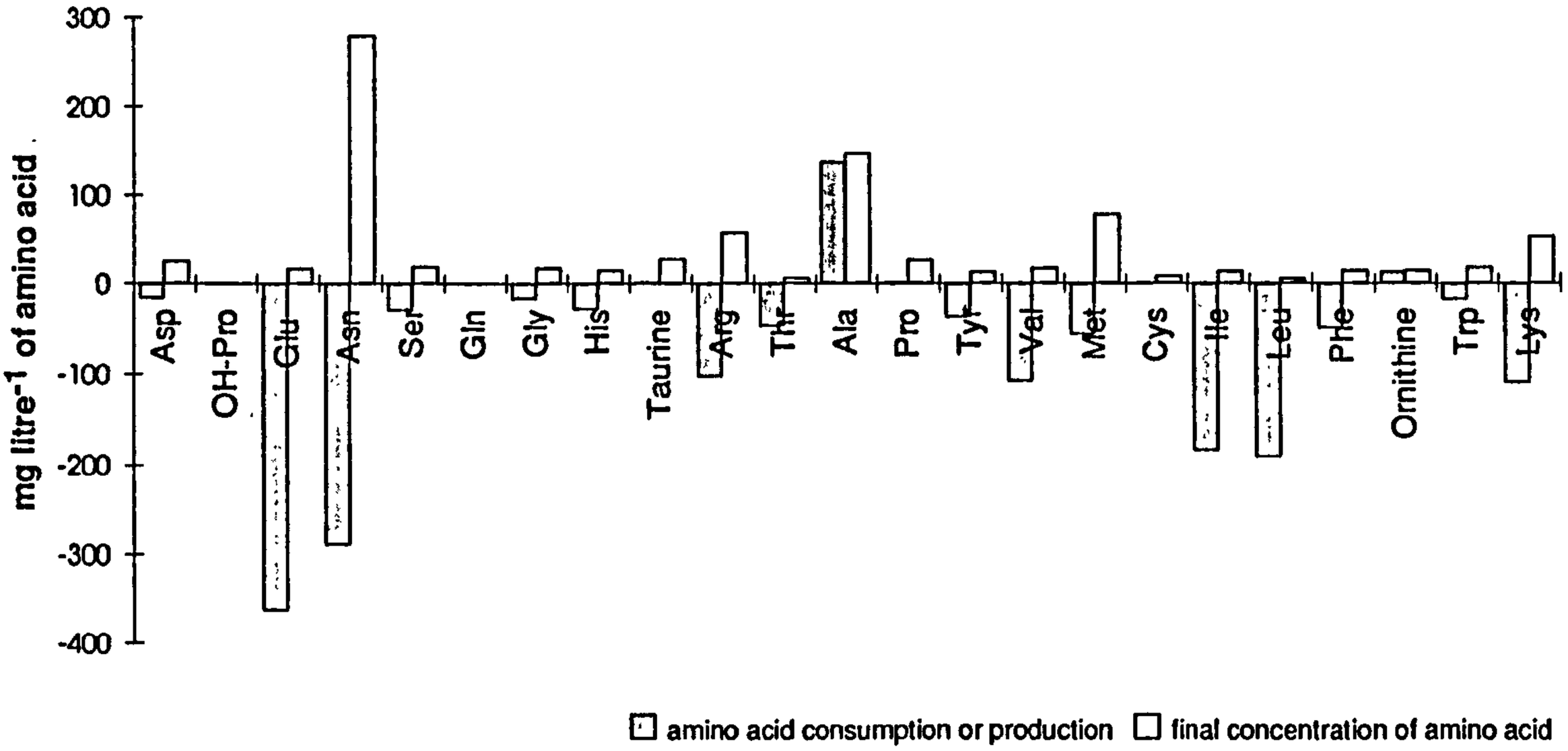

Fig. 3. Final concentration of amino acids and amino acid utilisation by NS0 9D4.CF cells cultured in shake flask using WNSD medium with recombinant insulin.

Antibody produced in serum-free medium is functionally active

CAMPATH-1H antibody produced in static or shake flask cultures in serum-containing or serum-free medium was examined for functional activity by ADCC. All preparations were found to have functional activity at 0.02 μg ml^{-1}. Further studies relating to the glycosylation and biological activity of CAMPATH-1H expressed in different cell lines and grown under different culture conditions will be published shortly (Lifely *et al.*, 1995).

Discussion

There are considerable advantages in the use of media that do not contain serum and that are essentially protein-free. In this study we have shown that GS-engineered NS0 cells expressing humanised monoclonal antibody can be grown in a novel protein-free medium, WNSA medium, supplemented with cholesterol rich lipid and β-cyclodextrin. WNSA is a complex medium of defined composition, containing a number of additional trace elements and vitamins. Poor growth of GS-engineered NS0 cell lines expressing humanised monoclonal antibody in serum-containing 'select' medium and WNSA suggested that these media were sub-optimal.

The nutritional requirements of NS0 cells are complicated by NS0 having an essential requirement for exogenous cholesterol. In this respect NS0 are similar to the NS-1 cell line that is deficient in 3-ketosteroid reductase activity and unable to convert lathosterol to cholesterol (Sato *et al.*, 1987). As NS0 are derived from NS-1 it is likely that they also lack 3-ketosteroid reductase activity. In serum, cholesterol and other lipids are found attached to carrier proteins such as LDL, HDL and albumin. The use of phosphatidylcholine:cholesterol vesicles with a carbohydrate carrier β-cyclodextrin enabled these cells to grow moderately well in protein-free medium. In order to overcome the complications caused by the cholesterol requirement, GS-engineered NS0 cells were adapted to cholesterol-independence. Several cloned GS-engineered NS0 and NS0 hybridoma lines secreting monoclonal antibody have also been adapted to cholesterol-independent growth (data not shown), with a 100% success rate. This implies that the majority, if not all, of individual cells within the NS0 cells population contain the genetic information required to produce active 3-ketosteroid reductase, but perhaps in a repressed form.

Nutrient depletion studies using cholesterol-requiring engineered NS0 cell lines in WNSA medium identified a number of amino acids that were being almost completely consumed. Interestingly the amino acid utilisation by the cholesterol-independent cell lines was found to be practically identical to that of the

lines prior to adaptation (data not shown), suggesting that amino acid metabolism was similar. An enriched medium (WNSD) was developed containing additional amino acids, choline chloride and ribonucleosides. Cholesterol-independent 9D4.CF cells grown in shake flask culture with WNSD reached a density of 1.86×10^6 cells ml^{-1} accumulating 76.6 mg $litre^{-1}$ functionally active (by ADCC) CAMPATH-1H antibody. In addition 9D4.CF reached a density of over 2.5×10^6 cell ml^{-1} in a one litre stirred bioreactor using WNSD supplemented with 0.1% w/v Pluronic F68 (data not shown). In WNSA medium it was found that human recombinant insulin (Nucellin) slightly improved cell growth, but that in WNSD Nucellin was not required. Amino acid analysis of spent WNSD culture supernatant showed that certain amino acids were still being fully depleted. A modification of WNSD medium which contained additional nutrients and a lower concentration of sodium salts (to adjust the osmolality) was toxic and inhibited cell growth. Future studies should concentrate on different feeding regimes to increase biomass, accumulated antibody and cell viability and on the prevention of cell death.

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Engineered Anti-CD38 Monoclonal Antibodies for Immunotherapy of Multiple Myeloma

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Multiple myeloma is a malignancy of plasma cells for which there is no effective treatment. To develop an immunotherapeutic agent, we have raised a high affinity mAb (AT13/5) against CD38, one of the few well-characterized surface Ags present on myeloma cells. Since murine monoclonals have many disadvantages as human therapeutics, we prepared two engineered forms of the Ab: a CDR-grafted humanized IgG1 and a chimeric $FabFc_2$ (mouse Fab cross-linked to two human γ_1 Fc). To retain affinity in the humanized Ab, a number of changes were required to the human framework regions of the heavy chain. In particular, through systematic mutagenesis and computer modeling, we identified a critical interaction between the side chains of residues 29 and 78, which may be important for the humanization of other Abs. The properties of the humanized IgG1 and $FabFc_2$ constructs were compared in a series of in vitro tests. Both constructs efficiently directed Ab-dependent cellular cytotoxicity against CD38-positive cell lines, but C was activated only poorly. Neither construct caused down-modulation of CD38, nor did they affect the NADase activity of CD38. Despite their differing structures, both Abs showed similar activity in most assays, although the humanized IgG1 was more potent at inducing monocyte cytotoxicity. These data represent the first direct comparison of CDR-grafted and chimeric $FabFc_2$ forms of the same Ab, and offer no support for the perceived advantages of the $FabFc_2$. These Abs show promise for therapy of multiple myeloma and other diseases involving CD38-positive cells. *The Journal of Immunology,* 1995, 155: 925–937.

Multiple myeloma is a fatal neoplasm characterized by an accumulation of a clone of plasma cells, frequently accompanied by the secretion of Ig chains. Bone marrow invasion by the tumor is associated with anemia, hypogammaglobinemia, and granulocytopenia with concomitant bacterial infections. An abnormal cytokine environment, principally raised IL-6 levels, often results in increased osteoclasis leading to bone pain, fractures, and hypercalcemia.

A variety of chemotherapeutic protocols have been tried over recent years with little impact on the overall prognosis for myeloma patients (1). As an alternative approach, several workers have proposed immunotherapeutic strategies targeting the IL-6-signaling system. IL-6 has been suggested to be a major growth factor for myeloma cells (2, 3) functioning in either an autocrine (4, 5) or paracrine fashion (6). Two murine monoclonals that neutralize IL-6 suppressed the proliferation of myeloma cells in a patient with a leukemic variant of the disease (7), although the tumor relapsed after 60 days. Similarly, the IL-6R has been investigated as a target for both blocking Abs (8) and IL-6-cytotoxin conjugates (9–12). A humanized anti-IL-6R Ab has been prepared for possible human therapeutic use (13), but its utility in myeloma patients remains to be demonstrated.

Our preference is for a more direct approach, targeting myeloma cells for killing by the host immune system. The surface Ag CD38 (14) is strongly expressed by a large majority of multiple myeloma cells (15–17) and its suitability as a target for lytic immunotherapy has been discussed (18). The same report also demonstrated the competence of effector cells from myeloma patients to lyse target cells coated with a chimeric anti-CD38.

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A prerequisite of such a strategy is that the therapeutic Ab is both maximally efficient in engaging human effector mechanisms and also acceptable to the host immune system. Conventional Ab derivatives, such as chimeric (19–21) and CDR[2]-grafted (22, 23) Abs, offer the potential for reduced immunogenicity compared with murine monoclonals, and have a spectrum of effector functions determined by their isotype. However, straightforward CDR-grafting often results in a drop in Ag-binding affinity, requiring changes to the framework regions to restore activity (23–28). Despite attempts to identify key framework residues of general importance for successful grafting (28–31), and the exploitation of computer models (24–26, 32) selection of appropriate mutations remains essentially an iterative experimental procedure required for each individual Ig.

Unnatural combinations of Ig domains have also been produced in attempts to produce molecules with enhanced effector function (33–39). One such molecule with particularly attractive potential is the $FabFc_2$—a monovalent Fab arm from a murine monoclonal chemically cross-linked to two Fc fragments of human IgG1. Such a construct offers the possibility of a weaker down-modulatory effect on surface Ag (40) while presenting a dual Fc array to effector cells—a feature that may enhance effector function (33).

In this report we describe the preparation of a humanized IgG1 monoclonal with specificity for anti-CD38. Through computer modeling and systematic mutagenesis, we identify an important heavy chain framework residue interaction required for full binding and effector activity. We also present the first direct comparison of the properties of a humanized IgG1 and a $FabFc_2$ with the same Id in a variety of functional assays.

Materials and Methods

Restriction and modification enzymes were purchased from New England Biolabs (Beverly, MA) and Boehringer Mannheim (Mannheim, FRG). AmpliTaq polymerase was from Perkin-Elmer Cetus (Norwalk, CT). Chemicals were obtained from Sigma Chemical Co. (Poole, UK), as were ELISA reagents. U1966 myeloma cells were a kind gift of Dr. K. Nilsson (University of Uppsala, Sweden). Monoclonal IB4 (41) was a kind gift from Dr. F. Malavasi (University of Turin, Italy). Anti-CD38 monoclonal B-A6 was from Serotec (Oxford, UK) and HB7 was from Becton Dickinson (Mountain View, CA). OKT10 hybridoma cells were obtained from the American Type Culture Collection (Bethesda, MA). FACS detection reagents were from Becton Dickinson and Sigma Chemical Co.

Immunization and production of the mouse monoclonal

The hybridoma AT13/5 was produced by standard hybridoma technology. Briefly, a BALB/c mouse was immunized s.c. at four sites with 5×10^6 Namalwa cells emulsified in CFA (Difco, Detroit, MI). Three weeks later, the animal was boosted with an i.p. injection of 5×10^6 Namalwa cells in IFA. A second i.p. boost was given at 4 mo, consisting of the same dose of cells in PBS. Three days after the final boost, a single cell suspension was prepared from the spleen and fused at a ratio of 1.6:1 with the mouse myeloma line NS1 (P3-NS-1/1-Ag 4.1). Hybridoma cells were screened after 10 days by flow cytometry against Namalwa cells, and clonal lines obtained by limiting dilution cloning.

Chemically engineered Abs

The chimeric Ab derivative $FabFc_2$ was prepared by thioether-linking hinge cysteines of mouse monoclonal Fab′γ with hinge cysteines of normal human Fcγ (33). Mouse IgG1 Ab AT13/5 was digested by pepsin to provide $F(ab'\gamma)_2$, which upon reduction with DTT yielded $Fab'(SH)_5$. Human IgG in the form of cold ethanol precipitate (Bio-Products Laboratory, Elstree, Herts, UK) was further purified by anion-exchange chromatography on DEAE-SepharoseFF (Pharmacia Biotechnology, St. Albans, Herts, UK) discarding the most acidic third of the protein. The resulting basic IgG was digested briefly by papain (50 μg/ml, pH 6.6, 30 min at 37°C) to yield Fcγ1: radial immunodiffusion revealed Fcγ2 at <1% of total protein and failed to detect Fcγ3 or Fcγ4. The Fcγ1 was then subjected to reduction, temporary blocking of one hinge SH by SS-bonding to plasma albumin while the contralateral SH was alkylated, and further SS-interchange to yield $Fc(SH)_1$ (42). Reaction with a 20-fold molar surplus of the bismaleimide linker *o*-phenylenedimaleimide (Aldrich Chemical Company, Poole, Dorset, UK) led to Fc-maleimide.

$Fab(SH)_5$ and Fc-maleimide, at a molar ratio of 1:2.7 and a total protein concentration of 2 to 3 mg/ml, were allowed to react at pH 5.0 and 25°C for 18 h. The proteins were concentrated by a cycle of absorption/desorption on the cation exchanger S-SepharoseFF (Pharmacia), and the major product $FabFc_2$ was purified by recycling chromatography on Superdex 200 (Pharmacia).

cDNA cloning

cDNA was prepared from 5×10^6 AT13/5 cells using the Micro Fast Track (Invitrogen; San Diego, CA) and Superscript Preamplification (Life Technologies, Inc., Paisley, Scotland) systems. The heavy chain V region was cloned by a PCR strategy using primers hybridizing to the signal peptide and proximal CH1 portions of the heavy chain message (43). The light chain V region was amplified using the primers MKVRev (43) and VK1-BACK (44). Reaction products were cloned into pUC18 for sequencing by the dideoxy terminator method using Sequenase v2 (USB; Cleveland, OH).

Humanization

Humanized V regions were constructed by a recombinant PCR technique (45). The general strategy has been described previously (46). In this approach, a *Spe*I site is introduced into the J domain of the humanized heavy chain, allowing the V region to be fused to an appropriately-engineered human C region cDNA cassette. The primers used for humanization of the heavy chain were:

A_H: 5′ GATCAAGCTTTACAGTTACTCAGCACACAG 3′
B_H: 5′ GTGGACACCATAACTGGTGAAGGTGAAGCC 3′
C_H: 5′ AGTTATGGTGTCCACTGGGTGAGACAGCCA 3′
D_H: 5′ TTGTAGTCTGTGCTTCCACCTCTCCACATCACTCCAATCCACTCAAG 3′
E_H: 5′ GAAGCACAGACTACAATGCAGCTTTCATGTCCAGAGTGACAATGCTG 3′
F_H: 5′ GGAGTCCATCACGAAGCCGGTCGTTATCATGGATTTTGCACAATAATAGAC 3′
G_H: 5′ AAATCCATGATAACGACCGGCTTCGTGATGGACTCCTGGGGTCAAGGCTCACTAGTCACAGTCTCCTCAGCC 3′
H_H: 5′ TAGAGTCCTGAGGGAATTCGGACAGCCGGGAAGGTG 3′

The humanized heavy chain V region is produced as a 650-bp fragment, which is cleaved with *Hin*dIII and *Spe*I to allow fusion with the C region. The template used for recombinant PCR of the heavy chain V region was Campath-1H heavy chain cDNA (47). The primers used for the humanization of the light chain were:

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[2] Abbreviations used in this paper: CDR, complementarity-determining region; ADCC, Ab-dependent cellular cytotoxicity; CDRH1, complementarity-determining region 1 of the heavy chain; $FabFc_2$, monovalent Fab arm from a murine monoclonal chemically cross-linked to two Fc fragments of human IgG1; CHO, Chinese hamster ovary.

A_L: 5′ GATCAAGCTTCTCTACAGTTACTGAGCACA 3′
B_L: 5′ CCGATTATATATGTCCTCACTTGCCTTACAGGTGATG GTCAC 3′
C_L: 5′ AGTGAGGACATATATAATCGGTTAACCTGGTACCAGC AGAAG 3′
D_L: 5′ AGTTTCCAAACTGGTTGCACCAGAGATCAGCAGCTTT GG 3′
E_L: 5′ GGTGCAACCAGTTTGGAAACTGGTGTGCCAAGCAGA 3′
F_L: 5′ GTACGGATTACTCCAATACTGTTGGCAGTAGTAGGTG GC 3′
G_L: 5′ CAGTATTGGAGTAATCCGTACACGTTCGGCCAAGGGA CC 3′
H_L: 5′ GATCAAGCTTCTAACACTCTCCCCTGTTGA 3′

The humanized light chain is produced as a 750-bp fragment encoding the entire human κ light chain. Primer nomenclature is according to Sims et al. (46). The template for the light chain humanization was Campath-1H light chain cDNA (47).

A chimeric heavy chain was produced by amplifying the murine V_H with the primers BL0 (GTACAAGCTTCAGGACCTCACCATGGCT GTGTTTAGCGCTG) and BL1 (GATCGACTAGTGTTCCTTGAC CCCAGGA), which insert restriction sites in the 5′ untranslated sequence and the J sequence, allowing fusion with the human C region cassette.

Variant humanized heavy chains containing additional murine framework residues were generated by recombinant PCR using the humanized chains as templates. These reactions used primers A_H and H_H together with a pair of primers encoding the desired mutation: residues 67–71, primers I_H and J_H; residues 28–29, primers K_H and L_H; residue 76, primers $M1_H$ or $M2_H$ (for wild-type and mutant 67–71 templates, respectively) and N_H; residue 78, $O1_H$ or $O2_H$ (for wild-type and mutant 67–71 templates, respectively) and P_H.

I_H: 5′ GTTGTCCTTGGTGATGTTCAGTCTGGACATGAAAGCTG C 3′
J_H: 5′ CTGAACATCACCAAGGACAACAGCAAGAACCAGTTC AGC 3′
K_H: 5′ ACTGGTTAACGAAAAGCCAGACACGGTGCAGGTCAG 3′
L_H: 5′ GGCTTTTCGTTAACCAGTTATGGTGTCCACTGGGTG 3′
$M1_H$: 5′ AAATTGCCGTTTCGAAGTGTCTACCAGCATTGTCAC 3′
$M2_H$: 5′ AAATTGCCGTTTCGAATTGTCCTTGGTGATGTTCAG 3′
N_H: 5′ TTCGAAACGGCAATTTAGCTTGAGACTCAGCAGC 3′
$O1_H$: 5′ TCTTAAGCTCACCTGGTTCTTGCTGGTGTCTAC 3′
$O2_H$: 5′ TCTTAAGCTCACCTGGTTCTTGCTGTTGTCCTT 3′
P_H: 5′ AACCAGGTGAGCTTAAGACTCAGCAGCGTGACA 3′

Typically, recombinant PCR was performed using the following cycle definition: step 0: 94°C for 120 s; step 1: 94°C for 60 s; step 2: 50°C for 60 s; step 3: 72°C for 60 s, go to step 1, repeating this loop for 25 cycles; and step 4: 72°C for 10 min.

Expression of humanized antibodies

For expression, cDNA encoding V_H regions of humanized heavy chains were cloned into a derivative of pUC18 containing the distal portion of the human J domain and the γ1 C region, to regenerate a complete heavy chain. Coding regions of the heavy and light chains were then transferred into pEE6.hCMV and pEE12 (Celltech, Slough, UK) (48, 49), respectively. The resulting plasmids were transfected into CHO DUK-B11 cells using Transfectam (Promega; Madison, WI) and culture supernatant was assayed for human Ig by ELISA after 3 days.

Large amounts of recombinant Ab were prepared by stable expression in NS0 or CHO cells. NS0 expression was performed as described (46). For stable CHO expression, the heavy and light chain genes were cloned into vectors pBAN2 and pRDN1, respectively (50). These plasmids were introduced into CHO B11 cells as described above. Selection, methotrexate amplification, and cloning procedures have been described (51). Stably transfected clones were grown in medium depleted of bovine Ig, and the recombinant Ab purified by protein A affinity chromatography.

Ab modeling

Ab Fv regions were modeled using the AbM package (Oxford Molecular Ltd., Oxford, UK), which implements a combined algorithm of homology and conformational generation techniques (52). Steepest descents energy minimization and constant temperature simulations (5000 s at 30 K) of solvated models were performed using SYBYL (Tripos, St. Louis, MO).

Flow cytometry

Cells for flow cytometry were washed in PBS + 5% FCS and resuspended to approximately 10^6/ml in the same buffer. Following the addition of Ab, cells were incubated at 4°C for 30 to 60 min and washed several times in PBS + 5% FCS. Bound Ab was visualized by incubating the cells with FITC-conjugates of anti-mouse IgG or anti-human IgG (Becton Dickinson and Sigma) for 30 min at 4°C. After washing, reactions were analyzed by flow cytometry using a FACScan (Becton Dickinson). FITC-labeled anti-CD38 (clone HB7; Becton Dickinson) was used as a positive control for CD38 expression. For inhibition studies, the first Ab was incubated with the cells for 45 min, and then the second Ab added without washing. After a further 45 min, the cells were washed and then visualized as above.

Ag modulation

A total of 1.6×10^5 U1966 cells were incubated in medium with dilutions of Ab at 37°C for 6 h. The cells were then washed and bound Ab was visualized with FITC-anti human antisera as above. The median fluorescence signal was calculated and compared with that obtained from duplicate control wells incubated with the same anti-CD38 for 45 min at 4°C.

Ab-dependent cellular cytotoxicity assays

The function of Abs in mediation of antibody-dependent cellular cytotoxicity was examined in a europium release assay (53–55). A total of 10^4 Wien 133 cells/well of a 96-well microtiter plate were labeled with europium-DTPA and then exposed to freshly prepared human PBL in the presence of Ab at an E:T ratio of 50:1. Lysis was estimated by determining release of europium after 4 h using a Delfia system (Wallac). Controls without Ab and/or effector cells were used to estimate spontaneous release. Maximum releasable europium was determined by Triton X-100 lysis of target cells. As a positive control, a humanized anti-CD52 mAb (Campath-1H; Wellcome Foundation Ltd.) was used.

Monocyte cytotoxicity

The ability of Abs to stimulate monocyte cytotoxicity was tested in a growth inhibition assay. Human peripheral blood monocytes were allowed to adhere to tissue-culture plastic for approximately 14 days, and assayed for CD14 expression by FACS. The cells were then harvested and dispensed at 10^5 cells/ml. A total of 10^4 monocytes were cultured with 4×10^3 Wien 133 target cells in the presence of Ab for 5 days. Target cell proliferation rate was then assessed by incorporation of [^{3}H]TdR. Growth suppression due to monocyte cytotoxicity was determined by reference to duplicate cultures without monocytes.

Complement-mediated lysis

A total of 5×10^5 target cells/well were labeled with europium and incubated for 4 h in medium containing 10% human serum in the presence of Ab. Determination of europium release was as for ADCC assays. Maximum releasable europium was determined by Triton X-100 lysis of target cells. Incubations without serum or without Ab were used to assess background release.

C1q binding

C1q binding was assessed by flow cytometry as described above. Briefly, 5×10^5 cells were stained with Ab at 4°C for 45 min. After washing, the cells were resuspended in medium containing 10% fresh human serum at 4°C for 30 min, washed briefly again, and then labeled with FITC-anti-C1q (Seratec) and visualized by FACScan. Ab binding was assessed in parallel experiments using FITC-anti-Ig reagents. As with the ADCC, Campath-1H was used as a positive control reagent.

NADase activity assays

These were performed essentially as described by Kontani et al. (56). A total of 2.5×10^5 cells were preblocked with 20 μg/ml Ab in medium for 30 min at 37°C, and then incubated with 100 μM 1,N^6-etheno-NAD (Sigma) in 100 μl PBS containing 10 μg/ml Ab for 15 min at 37°C. Twenty-five-microliter samples were taken and mixed with 50 μl 5% SDS and 1 ml sodium phosphate (pH 7.4). NADase activity was determined by fluorometry using an excitation wavelength of 300 nm, with emission intensity measured at 410 nm.

Results

Ab specificity

The specificity of the AT13/5 Ab was examined in a series of FACS experiments. The Ab stained Wien-133, U1966, and anti-CD3-stimulated human PBL, all of which express CD38 (data not shown). The Ab also stained CHO cells expressing human CD38 on their surface, but not wild-type CHO cells (Fig. 1).

The relationship between the AT13/5 epitope and those of other anti-CD38 monoclonals was also addressed by looking for inhibition of FACS staining. IB4, but not OKT10, could inhibit the binding of FITC-conjugated AT13/5 to CD38-positive cells (data not shown). Similar studies with a humanized derivative of AT13/5 showed inhibition of binding of two further anti-CD38 monoclonals, B-A6 and HB7 (data not shown).

Finally, AT13/5 immunoprecipitated a band of approximately 40 kDa, the m.w. of CD38. OKT10 precipitated a band of identical size from the same extract (Fig. 2). Taken together, these results demonstrate the specificity of the Ab.

cDNA cloning

Heavy and light chain V regions were cloned from the AT13/5 hybridoma by PCR. Several independent clones of each chain were sequenced on both strands and found to be identical. The sequences are given in Figure 3. The murine heavy chain uses a V gene from the Q52 family (Kabat group IB) and the J4 minigene. The light chain uses the J2 minigene and a V gene from the recently described Igk-V34 family (58).

Inadequacy of simple complementarity-determining region grafting

Human frameworks were chosen to receive the murine CDRs according to a best-fit homology strategy (46). The chains selected were REI (59) for the light chain, and NEW (60) with framework identities of 60% and 67%, respectively. The initial design of humanized Ab incorporated murine framework residues at positions 27, 30, and 94 of the heavy chain and position 49 of the light chain. The first two of these are residues shown to be important in another humanization using the same human chains (23). Residue 94 in the heavy chain is grafted as a standard part of the humanization strategy in our laboratory, and has been shown to be involved in the retention of binding activity (61). Light chain residue 49 is the final residue of FR2 and is substantially different in the AT13/5 and REI chains (Ser and Tyr, respectively). Humanized Ab genes were produced by recombinant PCR using Campath-1H heavy (NEW frameworks with murine residues at 27 and 30) and light (REI frameworks) chain cDNA clones (47) as templates.

On transient expression of the humanized chains in CHO cells, human Ig was secreted into the tissue culture medium, demonstrating association of the engineered heavy and light chains. However, the tissue-culture supernatant showed no significant reactivity for CD38-positive Wien 133 cells when analyzed by flow cytometry. Co-expression of the humanized light chain with a chimeric heavy chain (murine AT13/5 V_H fused to human Cγ1 at the engineered *Spe*I site) produced functional anti-CD38 Ab (here called hybrid Ab), indicating that the light chain had retained Ag-binding characteristics, but that the humanization of the heavy chain was inadequate.

Framework changes

To restore high-affinity binding in the humanized Ab, a systematic series of changes was made to the humanized heavy chain. Computer models of the mouse and humanized V regions were constructed using the AbM package (Oxford Molecular) and used in conjunction with sequence analysis to select four regions for further study: the stretch of residues from 66 to 73, residues 28 and 29, residue 76, and residue 78.

Variants of humanized heavy chain were prepared by PCR mutagenesis using the original humanized construct as template. Each heavy chain gene was co-expressed with humanized light chain in CHO cells, and the supernatants were assayed for binding to Wien 133 cells by FACS (Table I and Fig. 4). Of the 12 variants tested, four showed activity, although only one was comparable with the hybrid Ab (Fig. 5). This construct, h3S, was selected for further analysis.

Chemical chimera

Apart from humanization, other strategies have been proposed to enhance the utility of murine monoclonals as human therapeutics. In particular, chemical cross-linking of two human IgG1 Fc fragments to one murine Fab arm, to produce a monovalent $FabFc_2$ molecule, has been suggested to yield markedly enhanced effector function and reduced down-modulation of cell surface Ags (40).

To allow direct comparison of such a construct with a humanized IgG1 with the same specificity, a $FabFc_2$ was prepared from AT13/5 (data not shown), and compared with the h3S Ab in a variety of functional assays.

Modulation

One mechanism by which cells may escape Ab-directed lysis is by internalization or shedding of bound Ab-Ag

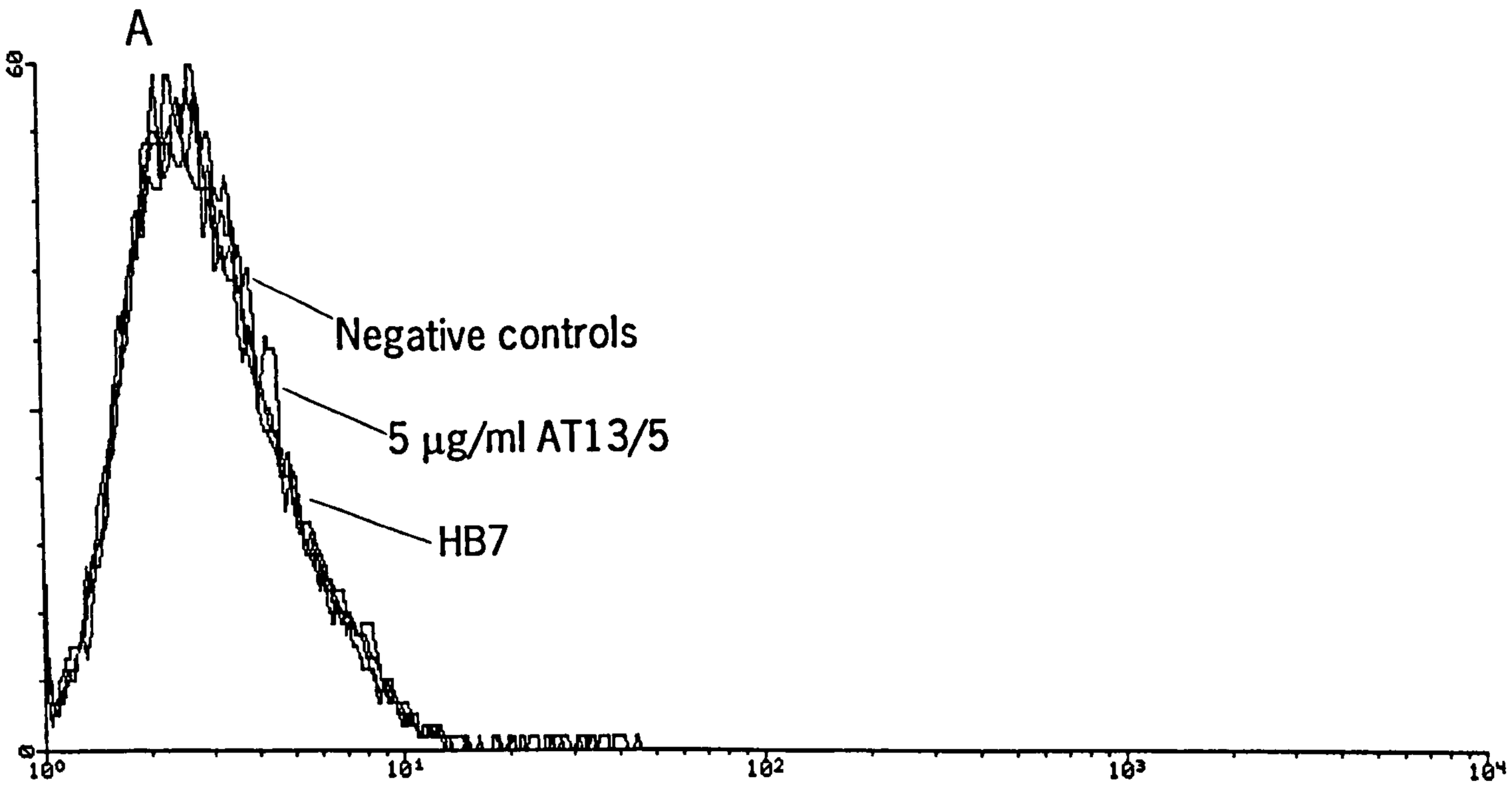


FIGURE 1. Specific staining by AT13/5 of CHO cells transfected with human CD38 as visualized by FACS. Untransfected CHO B11 (*A*) and CHO B11 transfected with CD38 (*B*) were stained with 5 μg/ml AT13/5 and HB7 (anti-human CD38, Becton Dickinson; concentration as recommended by manufacturer) and visualized with FITC-conjugated goat anti-mouse IgG serum.

complexes from the cell surface (down-modulation). The AT13/5 FabFc_2 and h3S Abs were compared for their ability to induce down-modulation of CD38 from the surface of U1966 myeloma cells. After 6-h incubation in the presence of Ab, there was no evidence for loss of Ag-Ab complexes from the cell surface (Fig. 6). A similar experiment, in which the cells were incubated with Ab for 24 h, yielded a similar result.

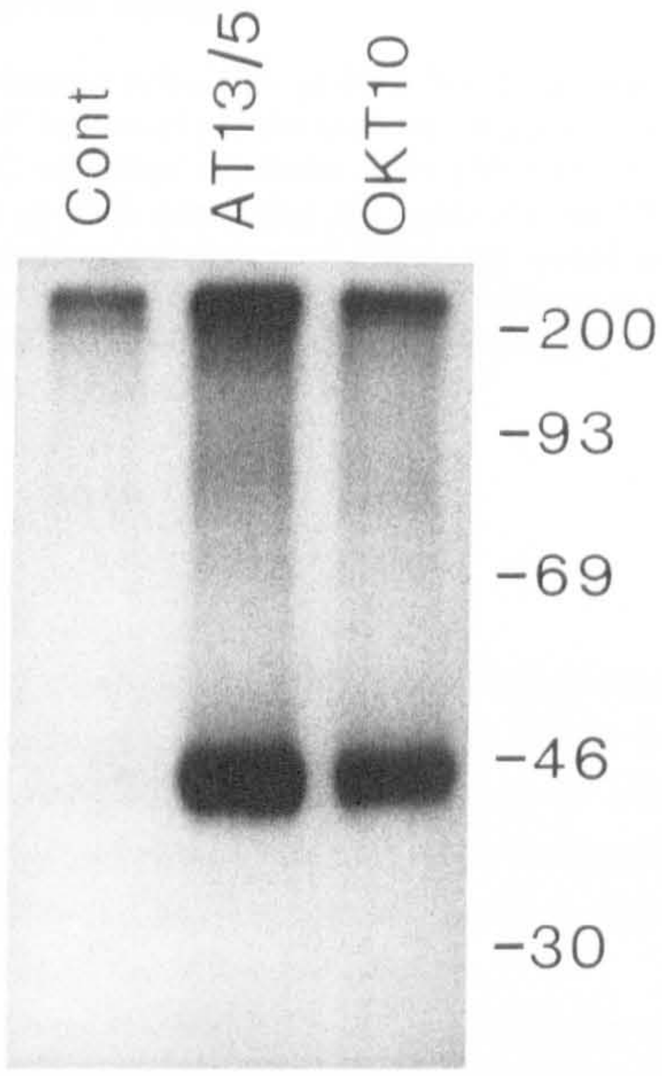


FIGURE 2. Immunoprecipitation of CD38. Namalwa cells were surface iodinated, solubilized, and immunoprecipitated as previously described (36), using hybridoma culture supernatant from a control (nonbinding mouse IgG1), AT13/5, or OKT10. Samples were analyzed on 12.5% SDS-gels under reducing conditions followed by autoradiography. Molecular weight markers are indicated in kilodaltons.

Effector function

To assess the biologic activity of the different engineered forms of the anti-CD38 Ab, direct comparisons were made in cytotoxicity assays using Wien 133 cells as targets. This cell line is of human lymphoid origin (62) and expresses both CD38 and CD52 at high levels, allowing comparison of the anti-CD38s with a highly-lytic humanized anti-CD52 monoclonal, Campath-1H (23). Also, as one of the main prospective targets for these engineered Abs are the tumor cells of multiple myeloma, cytotoxicity studies were also performed on the human myeloma-derived line U1966.

All forms of anti-CD38 were effective in killing Wien 133 cells by ADCC (Fig. 7), although Campath-1H was consistently three- to fivefold more potent at inducing killing. Half-maximal lysis was obtained with Ab concentrations in the low nanogram/milliliter range. Similarly, U1966 target cells were also killed by ADCC in the presence of engineered anti-CD38 Abs (data not shown).

In a preliminary experiment to determine whether the Abs were capable of directing C-mediated lysis, Wien 133 cells were exposed to hybrid and $FabFc_2$ Abs in the presence of human C. No lysis was observed, although FACS staining revealed that the target cells were labeled by the Abs. In a parallel experiment, Wien 133 cells were effectively killed by Campath-1H (data not shown), demonstrating that the cells are susceptible to lysis by human C.

Incubation of U1966 cells with humanized IgG1 and $FabFc_2$ Abs and human serum also failed to produce lysis. This was associated with an absence of C1q binding as determined by FACS staining (Fig. 8). To determine whether the failure to engage C was due to an intrinsic property of the Abs or to some unfavorable disposition of the Ag on the target cells, the C lysis assay was repeated using transfected CHO cells expressing high levels of surface CD38 (J. H. Ellis and K. A. Barber, unpublished data). Using these cells as targets, both C-mediated cytolysis and C1q binding were obtained using both anti-CD38 constructs (Fig. 8).

Finally, because a major in vivo mechanism for the clearance of Ab-coated target cells may be phagocytosis by cells of the monocyte lineage, the opsonizing effect of the Abs was examined. Both Abs induced monocyte toxicity toward Wien 133 target cells, although the humanized Ab was significantly more effective (Fig. 9).

Inhibition of NADase activity of CD38

Human CD38 has been shown to catalyze the production of cADPR from NAD (63, 64). cADPR has calcium-mobilizing activity and in one study (65) was shown to potentiate the proliferation of stimulated mouse lymphocytes. If this activity operates in vivo, it represents a possible auto-stimulatory pathway for CD38-positive cells. To determine whether the engineered anti-CD38 Abs might suppress such a signal, their effect on the CD38-catalyzed breakdown of a NAD analogue was measured by fluorometry. Neither the humanized Ab nor the $FabFc_2$ construct affected the CD38 NADase activity of transfected CHO cells (Fig. 10).

Discussion

Humanization

Although the process of transferring a specificity from one Ab molecule to another was originally conceived as being a graft primarily of the complementarity-determining regions (22, 66), it has become increasingly clear that a number of residues outside these domains exert a critical influence on the affinity of Ag binding. Certain positions in the heavy chain frameworks seem to be important in the retention of Ag-binding activity in a variety of different Abs (23, 25, 26, 28, 30, 61). In particular, many investigators have reported the grafting of donor residues at positions 67, 69, 71, and 73. These residues form a β-sheet in contact with the interior aspect of the CDRH2 loop: presumably mismatches at these positions distort the CDR shape. Also, donor residues at positions 91 and 94 appear to be required for correct CDRH3 conformation in many V_H.

In addition to these general observations, there may be other framework residues that are important in particular classes of V_H structure or even individual Abs. The AT13/5 V_H illustrates this point, in that despite incorporation of the framework modifications described above,

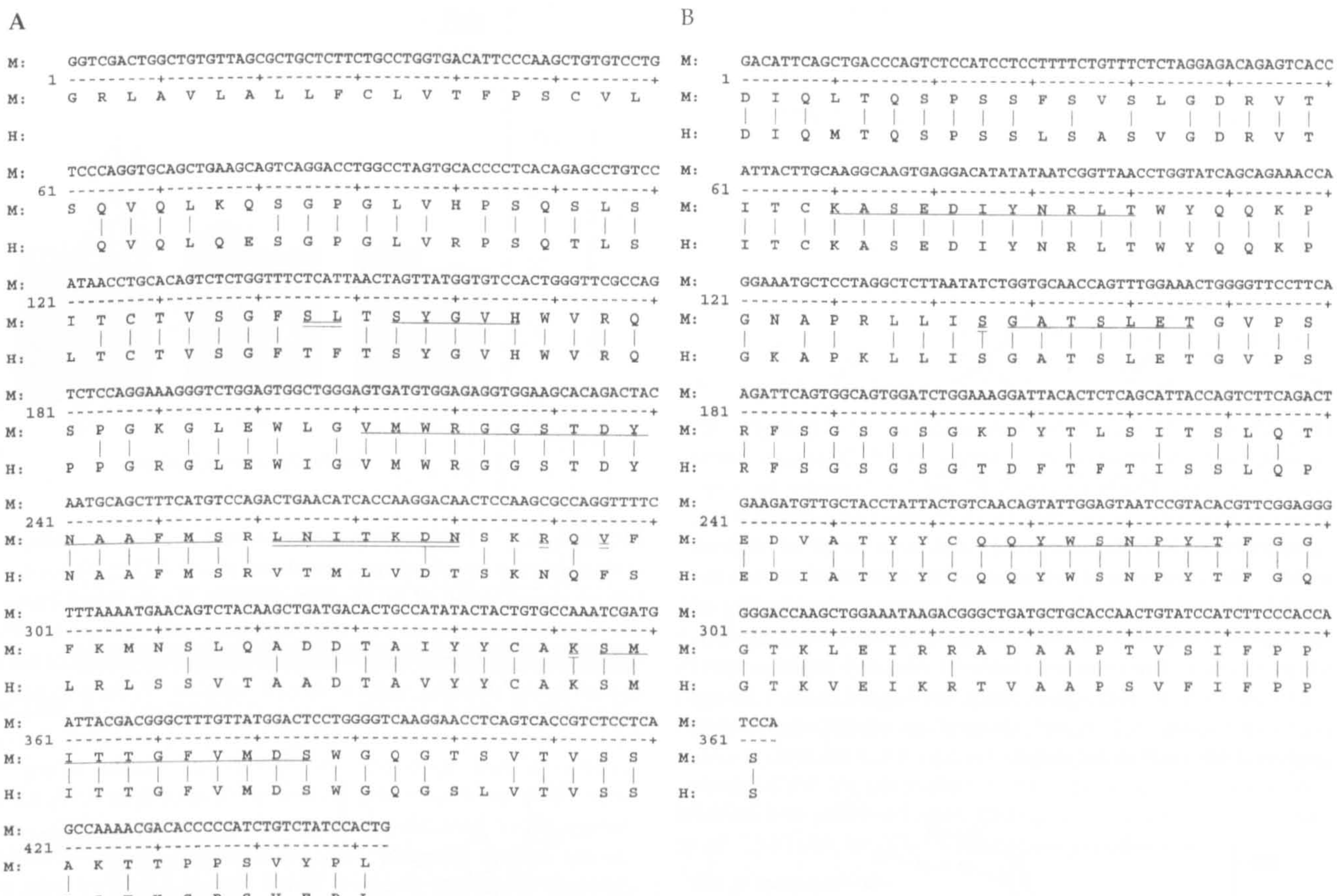


FIGURE 3. cDNA and deduced amino acid sequences of the framework regions of the heavy (*A*) and light (*B*) chains of the AT13/5 Ab. CDR residues as defined by Kabat et al. (57) are underlined. Murine framework residues incorporated into one or more of the humanized chain variants are doubly underlined. The amino acid sequence of the initial design of humanized Ab is shown underneath the AT13/5 sequence, identity between the chains is indicated by vertical lines. There is 67% identity between the framework residues of the heavy chain and 60% identity between those of the light chain. The murine sequences reported here have been submitted to Genbank under the accession numbers U27110 and U27111.

Table I. *The effect of heavy chain framework substitutions on the binding of humanized anti-CD38 Ab to CD38-positive cells in FACS assays*[a]

Construct	67–73	28/29	76	78	Binding
h1	−	−	−	−	−
h2	+	−	−	−	−
h3J	−	+	−	−	−
h3K	+	+	−	−	+
h3L	−	−	+	−	−
h3M	+	−	+	−	−
h3N	−	+	+	−	−
h3O	+	+	+	−	+
h3P	−	−	−	+	−
h3Q	+	−	−	+	−
h3R	−	+	−	+	(+)
H3S	+	+	−	+	++

[a] In the body of the table, − denotes that the human residue is used at a framework position, + that the corresponding residue from the murine V_H was used. With regard to binding, − indicates no detectable binding, and (+), +, and ++, progressively stronger binding, as estimated by measuring the fluorescent signal produced by a determined quantity of antibody.

the initial humanized construct failed to bind Ag. To minimize the number of additional nonhuman residues required to regain binding, we have used a combination of sequence analysis, molecular modeling, and systematic mutagenesis to identify pivotal residues responsible for functional differences between the mouse and humanized V_Hs.

When the mouse and h2 humanized Fv domains were modeled using AbM alone, the CDRs of the two Abs exhibited identical conformations; however, experimental data demonstrated that the h2 humanized construct was without binding activity. Since incorporation of murine residues at positions 28 and 29 resulted in partial recovery of affinity, the modeling appeared to be incomplete. CDRH1, adjacent to these residues, was modeled as a class 1 canonical loop by AbM due to conservation of defined key residues (29). Residues 27–37 and 78 of the heavy chain were subjected to minimization and dynamics simulation, resulting in the conformations shown in Figure 11.

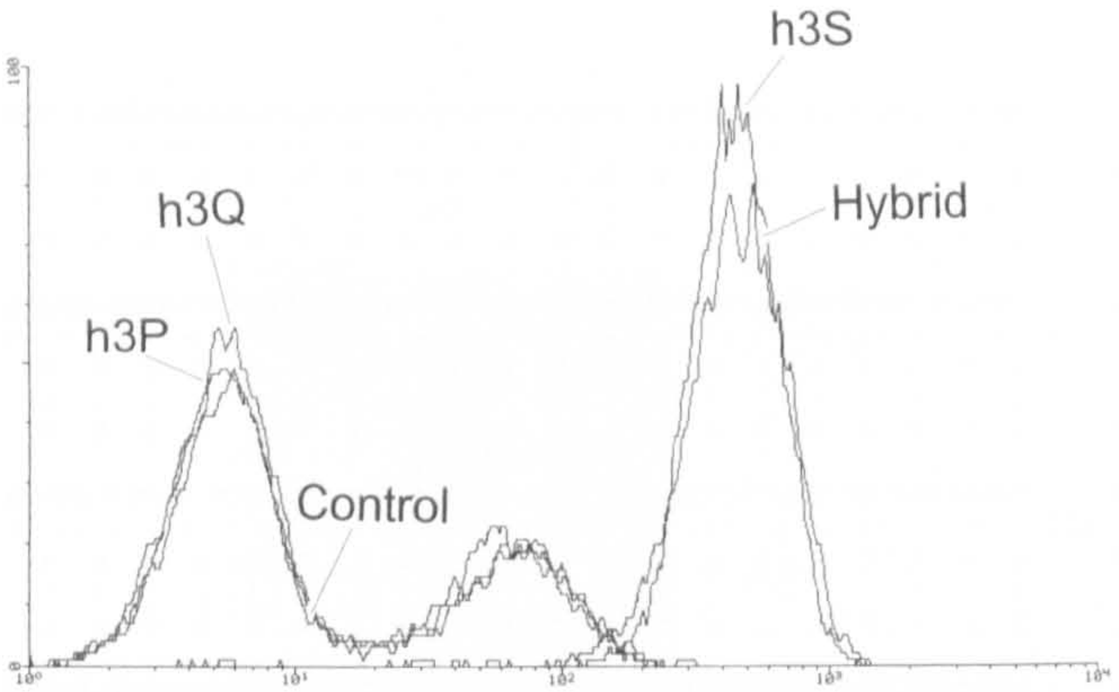


FIGURE 4. The CD38-binding activity of humanized Ab variants was determined in a series of FACS assays using Wien 133 cells. The traces for h3P and h3Q overlie the negative control data, as did the traces for the other nonbinding variants h1, h2, h3J, h3L, h3M, and h3N in other experiments. The control and nonbinding Ab distributions are bimodal: the smaller peak arises from nonspecific binding of the FITC-conjugated anti-human staining Ab to a subpopulation of cells. The concentrations of Ab used in this experiment were: h3P, 340 ng/ml; h3Q, 400 ng/ml; h3S, 125 ng/ml; and hybrid, 125 ng/ml. A total of 10,000 cells were analyzed for each distribution.

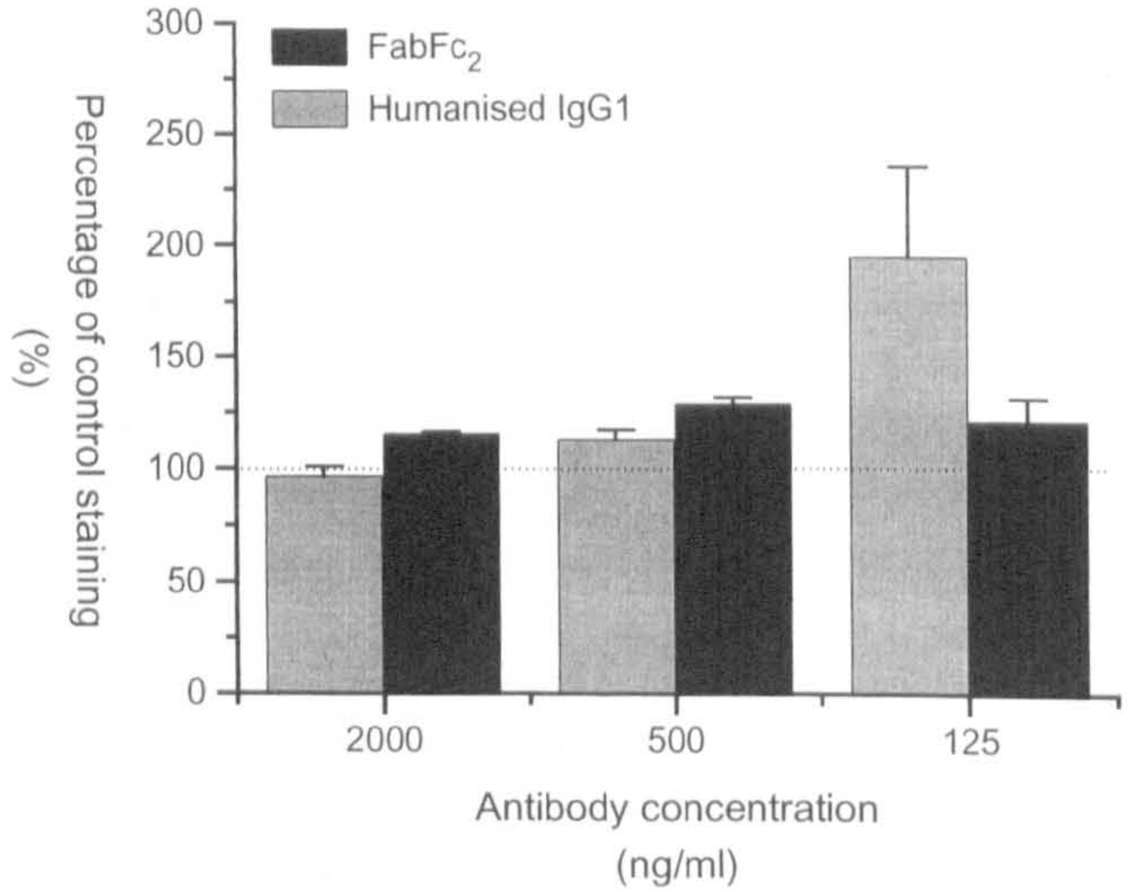


FIGURE 6. U1966 cells were incubated at 37°C in medium containing the specified concentrations of anti-CD38 Ab for 6 h before visualization of Ab binding. Median fluorescence intensity was normalized to that obtained from cell stained for 45 min at 4°C. Points represent the mean and standard error of two experiments, differing slightly in cell number. A third experiment with a 24-h incubation time gave similar results.

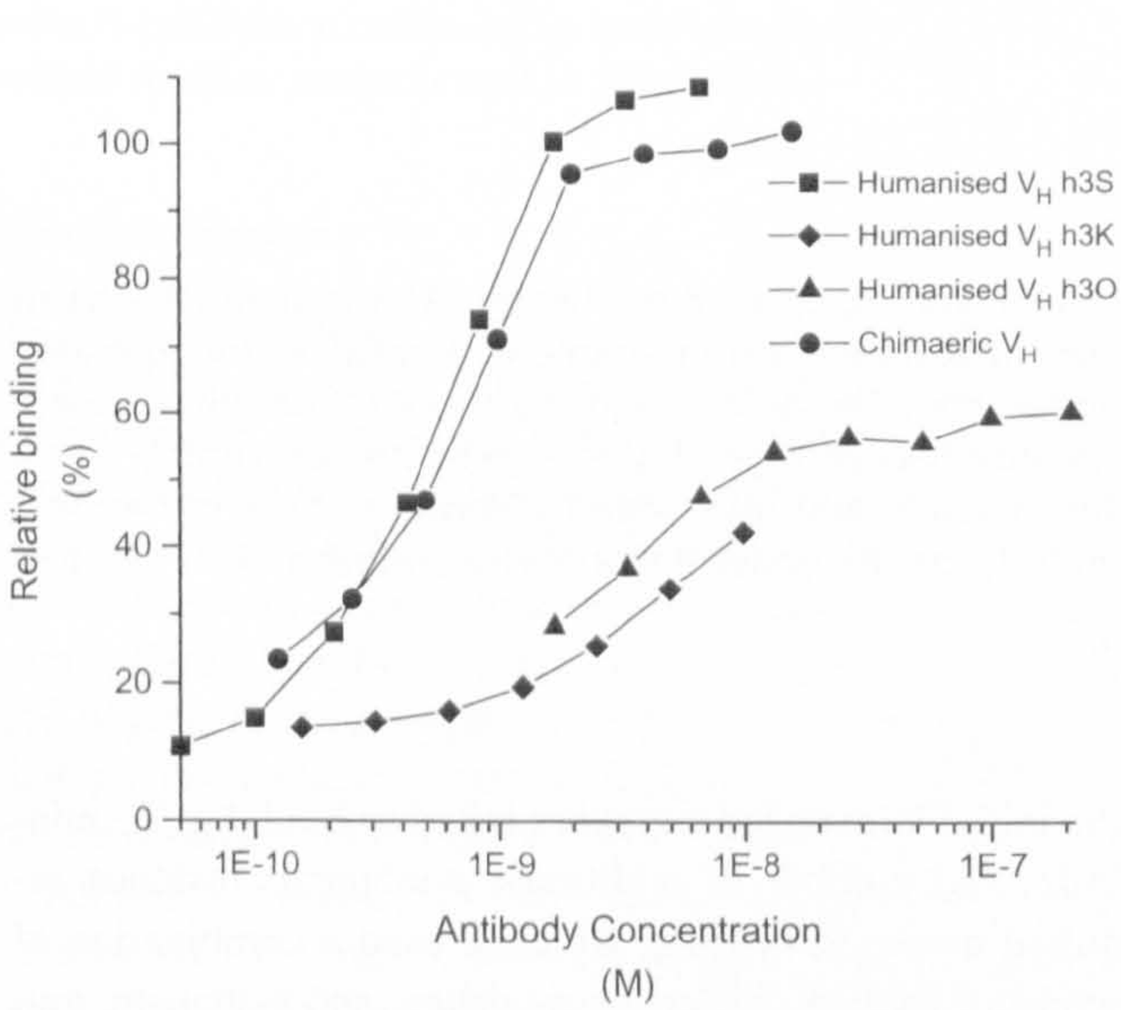


FIGURE 5. The relative binding activity of the active humanized heavy chain variants was determined in a FACS assay using Wien 133 cells. The mean fluorescence signal obtained from a dilution of Ab is plotted normalized to that obtained from a saturating amount of hybrid Ab. A total of 10,000 cells were analyzed for each distribution. The binding curve for the h3S construct indicates half-maximal binding at 6×10^{-10} M, giving an apparent K_a of 1.6×10^9 M^{-1}. This is in good agreement with a K_a of 3×10^9 M^{-1} obtained for AT13/5 by binding of iodinated Ab to Namalwa cells (unpublished observations).

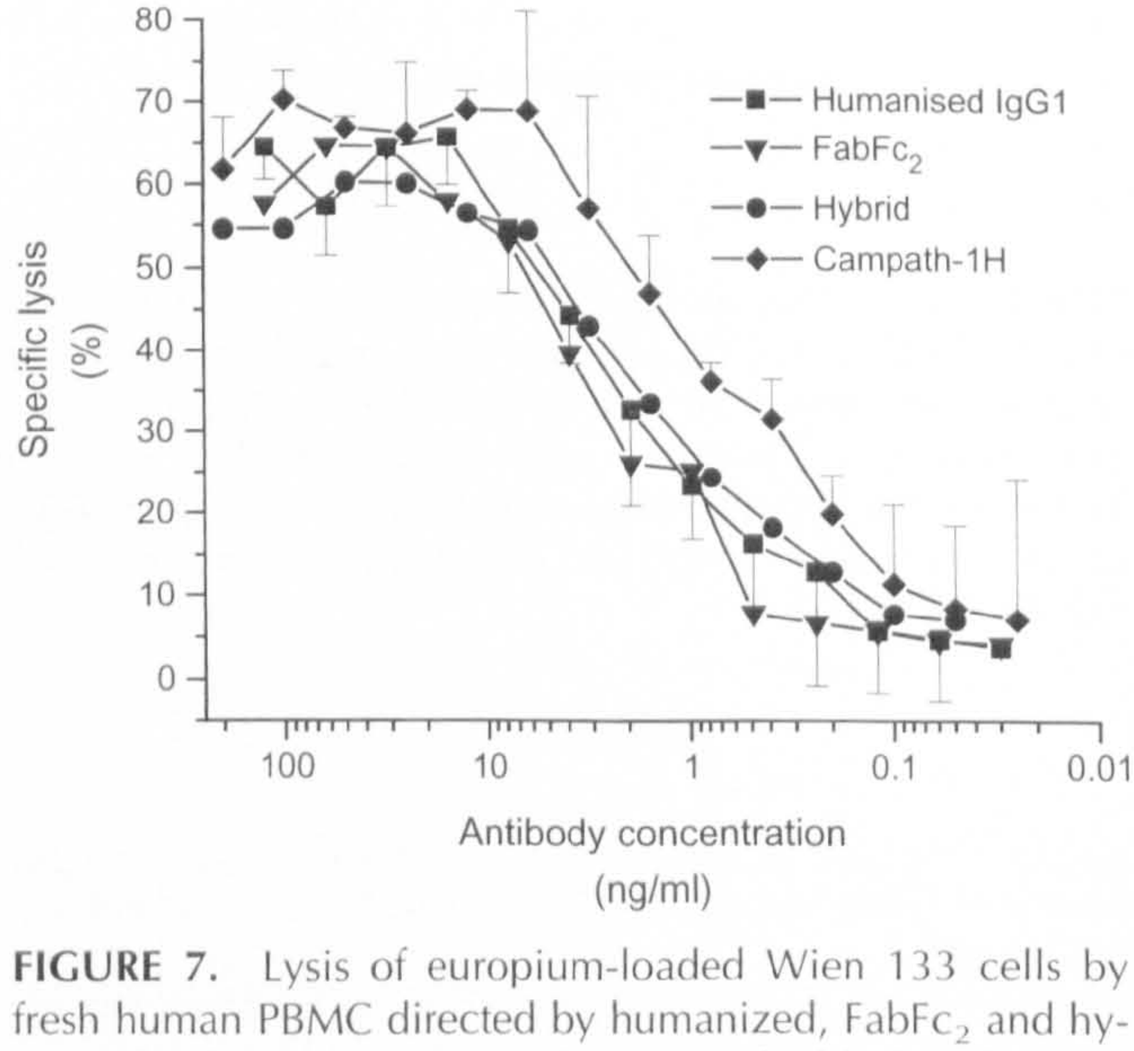


FIGURE 7. Lysis of europium-loaded Wien 133 cells by fresh human PBMC directed by humanized, $FabFc_2$ and hybrid anti-CD38 Abs. Campath-1H is a highly-lytic humanized anti-CD52 used here as a positive control for ADCC activity. Points are the mean and SE of triplicate determinations. For clarity, SE are shown for two of the four Abs: those for the remaining two are comparable.

Comparison of models for murine and humanized variable regions reveals an interaction between two framework residues that indirectly affects CDRH1. In the mouse V_H, the side chains of Leu-29 and Val-78 pack together in a small hydrophobic pocket close to CDRH1. In the human V_H selected as the graft recipient, the analogous positions are occupied by two Phe residues. It appears that the larger aromatic side chains are too bulky to pack in the same fashion, and so alter the disposition of neighboring surface residues, resulting in a different conformation of

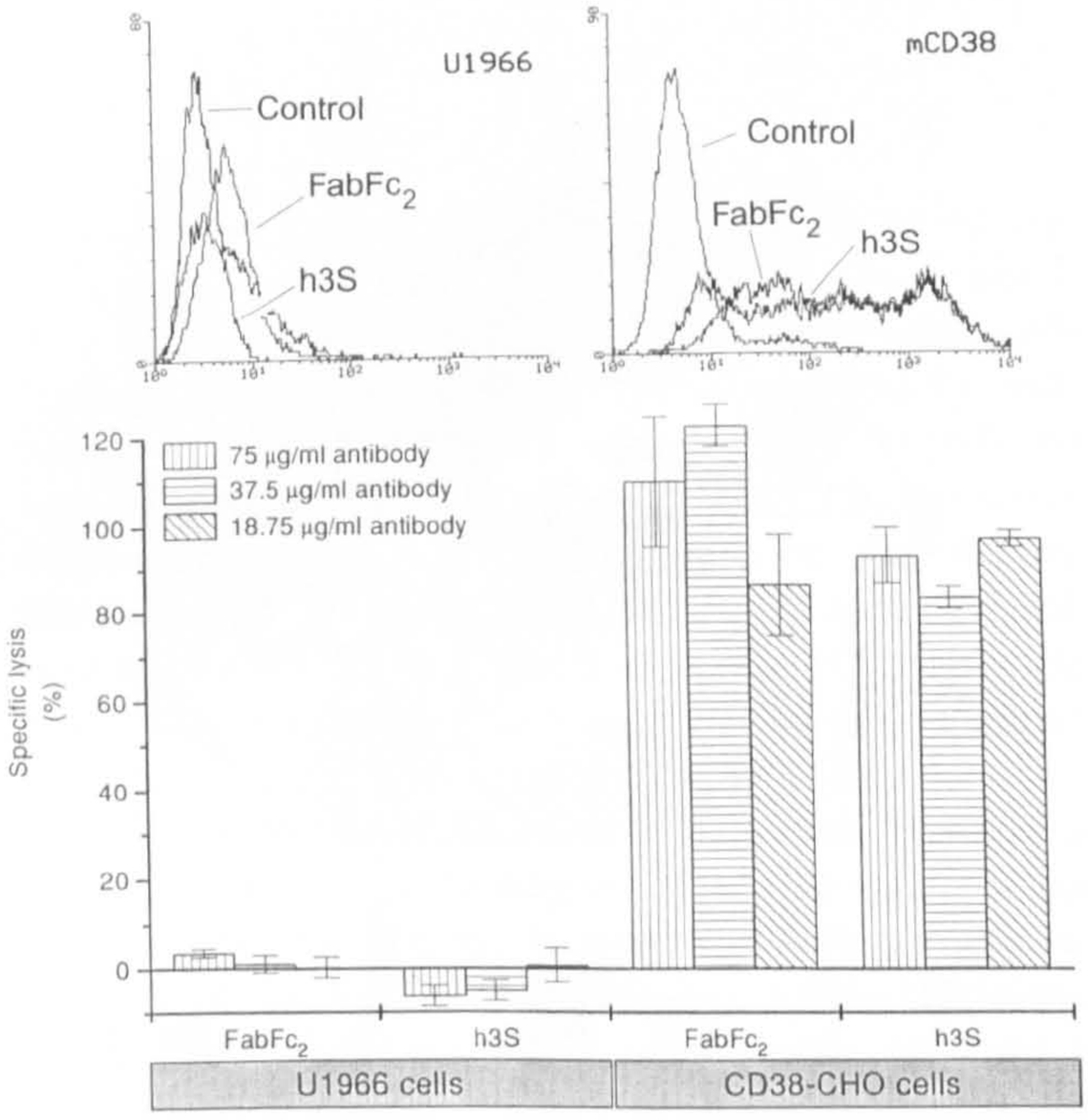


FIGURE 8. Engagement of C by anti-CD38 Abs as assessed by cell lysis and C1q binding. Cell lysis was determined by europium release: points represent the mean and SE of triplicate determinations. C1q binding was visualized in a FACS assay: the traces indicate the staining produced by 50 μg/ml of humanized IgG1 and $FabFc_2$ Abs. Negative control traces are also included on the FACS plots. In additional control experiments (not shown), an irrelevant humanized IgG1 neither bound C1q nor lysed the target cells, and no C1q binding was observed when the Abs were incubated with wild-type CHO cells.

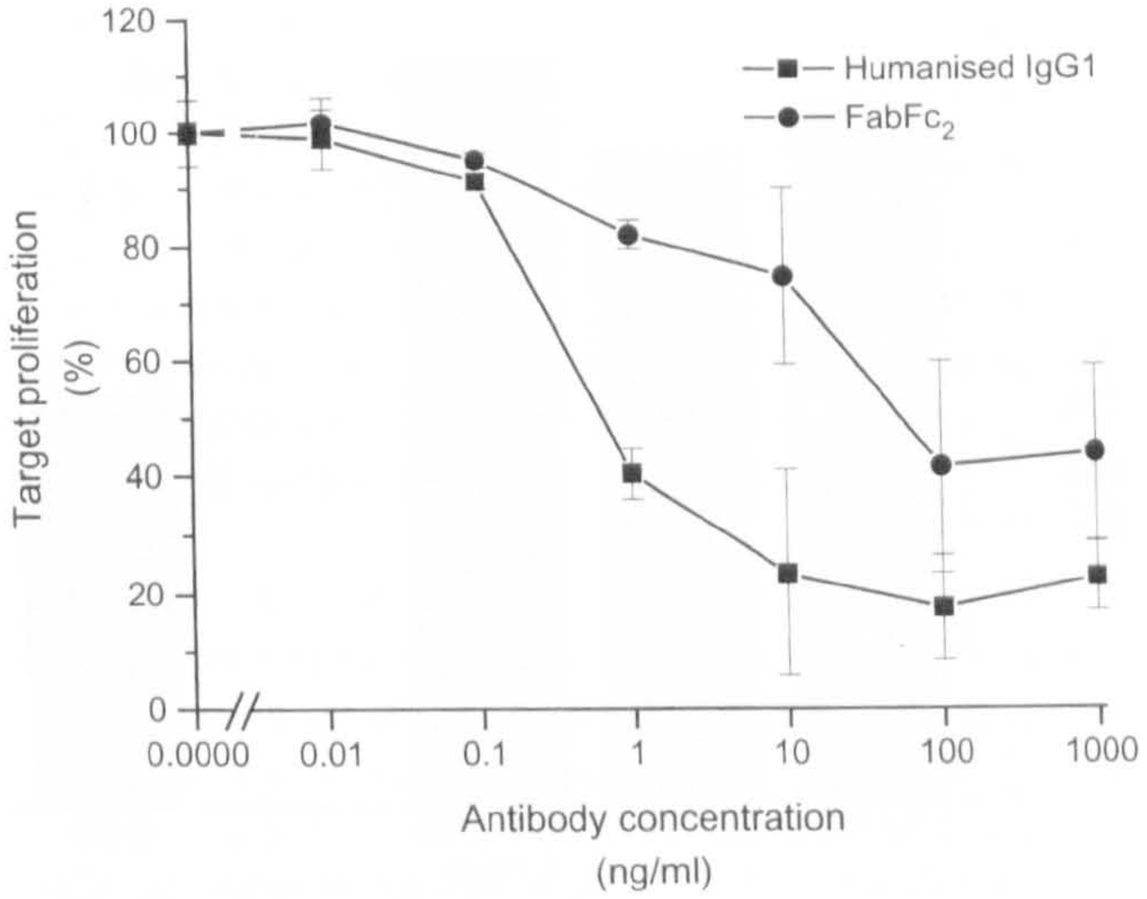


FIGURE 9. Proliferation of CD38-positive Wien 133 cells is suppressed by macrophages in the presence of anti-CD38 Abs. Proliferation was determined by incorporation of a [^{3}H]TdR pulse, and is expressed normalized to that obtained in the absence of Ab. Duplicate cultures lacking macrophages showed no dose-dependent inhibition of growth. Points are the mean and standard error of triplicate determinations. One hundred percent target cell proliferation corresponds to the incorporation of 332,000 cpm. Maximum inhibition was achieved with 100 ng/ml h3S, yielding a count of 58,594 cpm (17.4% of maximum proliferation).

CDRH1 (Fig. 11). Substitution of Phe-29 by the smaller murine residue partially relieves this effect, allowing Ag binding, albeit at reduced affinity. Full affinity was restored only by the replacement of both residues.

CDRH1 class 1 canonical loops have Ile, Phe, or Leu defined as a key residue at position 29, but do not specify residue 78. Analysis of the AbM Ab dataset revealed that all class 1 canonical loops in that database have nonaromatic hydrophobic residues (Ala, Leu, or Val) at position 78. The only Ab structure to have Phe residues at both positions 29 and 78 is 7FAB (NEW), which due to other key canonical residues has a CDRH1 with a class 2 canonical definition. Our data suggests that the combination of two Phe residues at positions 29 and 78 in a class 1 canonical environment may shift the CDRH1 conformation away from the class 1 canonical form.

The question arises as to whether these results have applicability to the humanization of other murine Abs or simply reflect an idiosyncrasy of the AT13/5 V_H structure. In support of the former view, we note that the AT13/5 V_H is a good match to the consensus for Kabat group IB murine V_H sequences and that within this group, Leu-29 and Val-78 are almost invariant. These data suggest that the requirement for small side chains at these positions may be a feature of a group of structures. Accordingly, we suggest that if CDRs from such V_Hs are to be grafted onto human frameworks containing bulky residues at these positions, then transfer of murine residues or similar small residues at 29 and 78 may be required to maintain the desired CDRH1 conformation.

With regard to the role of the AT13/5 light chain in Ag binding, our data do not allow any firm conclusions to be drawn. The similarity in the apparent affinities of the hybrid and parental murine Abs suggests that the humanized light chain possesses similar Ag-binding activity to the murine light chain; however, we cannot exclude the possibility that the properties of the light chain are simply of minor importance in determining the binding of the AT13/5 epitope.

Functional studies

We have compared the performance of a humanized IgG1 and a $FabFc_2$ construct in in vitro functional assays. Both feature the same high-affinity CD38-binding site, but offer potential differences in other aspects of their interaction with target cells and effector systems. Given the markedly different structures of the humanized IgG1 and $FabFc_2$ constructs, it might be expected that substantial functional differences may be observed; however, in the present experiments, both Abs showed broadly comparable activity,

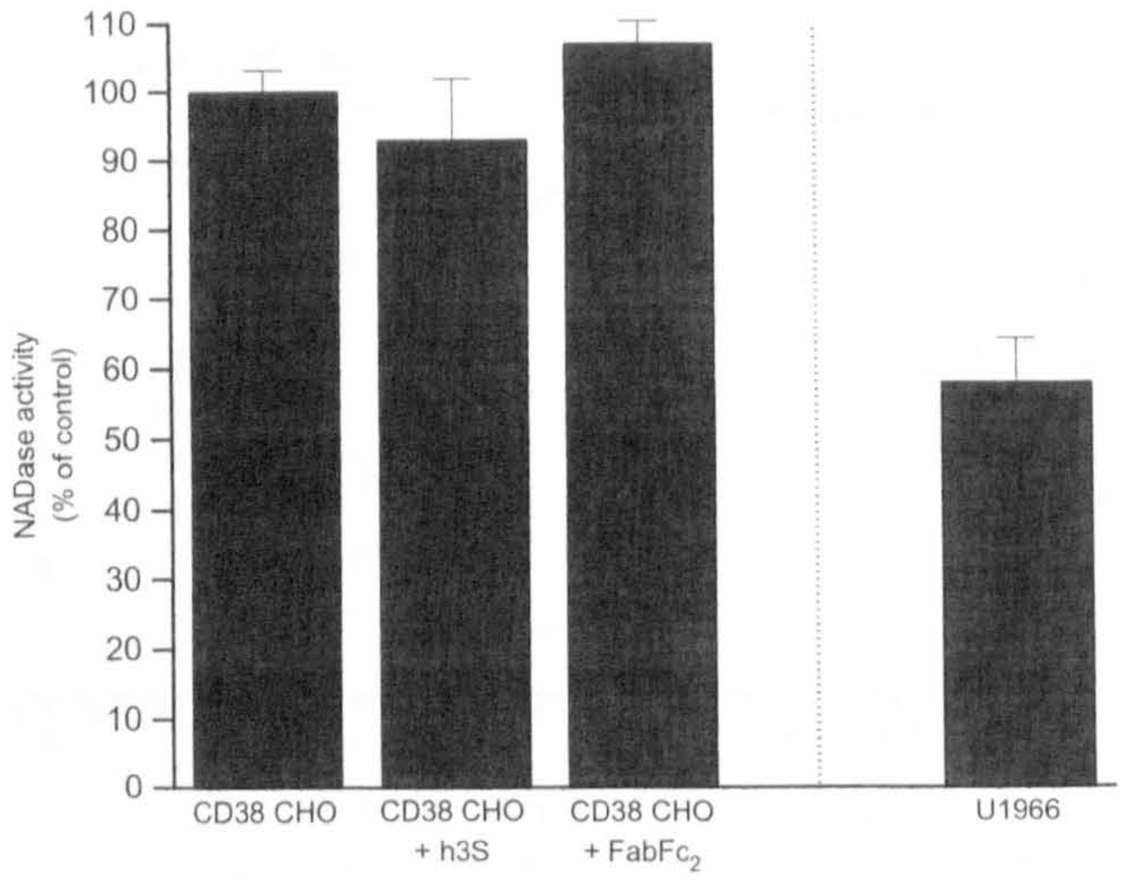
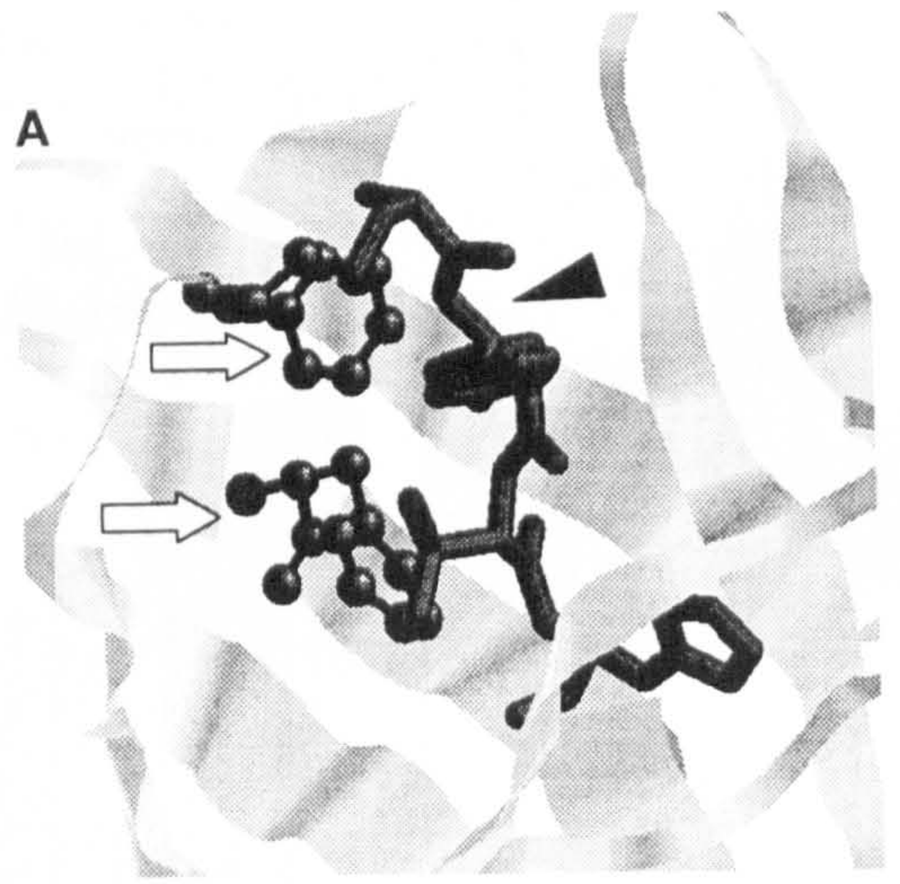


FIGURE 10. The NADase activity of CHO cells transfected with human CD38 is unaffected by incubation with 10 μg/ml of humanized or $FabFc_2$ Ab. Points are the mean of triplicate determinations, error bars indicate SE. Fluorescence signal was corrected for background activity using duplicate samples where CD38-positive CHO B11 cells were replaced with untransfected B11 cells, and normalized to the value obtained with CD38-CHO cells in the absence of Ab (background activity, 374 fluorescence units; control CD38-CHO, 464 U = 100%). The sensitivity of the assay to CD38 function is indicated by the value obtained using U1966 cells, which have a lower surface expression of CD38 than the CHO transfectants.

being strongly cytotoxic for CD38-positive cells in the presence of effector cells.

Although the Abs are of the γ1 isotype, which is competent for C fixation, no C lysis of two human cell lines was obtained, even at high concentrations of Ab. This may reflect the operation of proteins with a protective function against autologous C, such as CD55 and CD59 (67). However, the failure of the anti-CD38 Abs to cause lysis was accompanied by an absence of C1q binding, a step not known to be inhibited by such proteins (68, 69). Also, Campath-1H lysed CD38-positive CD52-positive Wien 133 cells under the same conditions.

Alternatively, the AT13/5 epitope may offer an intrinsically poor target for Ab-directed C attack. To explore this possibility, high-expressing CD38-transfected CHO cells were tested in the C assay. Both C1q-binding and lysis were obtained, showing that the epitope per se is compatible with C activation. Although the transfectants do express CD38 at higher levels than the human cell lines, it seems unlikely that the results can be explained purely by Ag density, as Wien 133 and U1966 cells are strongly CD38 positive. Perhaps more likely is a favorable disposition of Ag on the transfectants, a variable known to affect C binding (70, 71). Other workers have also reported humanized IgG1 Abs lacking C1q-binding activity (46), and in at least one case, two such Abs against CD4 show markedly different activity (72, 73).



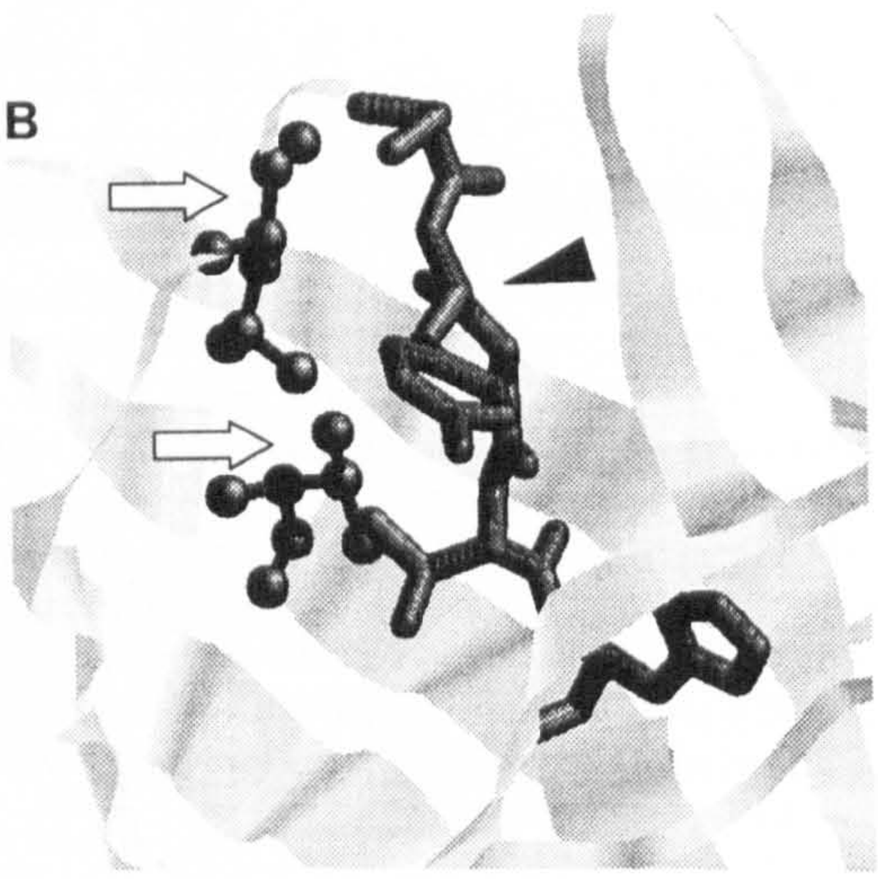


FIGURE 11. A detail from the computer models of the mouse (*A*) and humanized h2 (*B*) anti-CD38 V regions constructed using AbM (Oxford Molecular) and SYBYL (Tripos) and visualized using RasMol (R. Sayle; Glaxo, Middlesex, UK). Replacement of human frameworks containing *A*, Phe-29 and Phe-78 (ball-and-stick; white arrows) with *B*, residues Leu-29 and Val-78 (ball-and-stick; white arrows) relieves the distortion of CDRH1 (black arrowhead).

Although the dual Fc of the $FabFc_2$ construct has been reported to substantially improve C activation in comparison with a homologous FabFc derivative (42), a similar enhancement was not seen relative to the humanized IgG1 construct in this study. The $FabFc_2$ showed no superiority under suboptimal conditions for C activation (with the human cell lines as targets), or under permissive conditions. These findings are in accordance with those of Greenwood et al. (39), who found that incorporation of a tandem duplication of Fc in a derivative of a humanized anti-CD52 Ab had little effect on C activation.

In cell-mediated cytotoxicity assays, both the $FabFc_2$ and humanized IgG1 showed strong lytic potential, with lysis detected down to nanogram per milliliter concentrations of Ab. Both Abs recruited fresh human PBMC to kill

CD38-positive target cells with equivalent potency. However, in a second assay using purified monocytes as effector cells, the humanized IgG1 construct was clearly superior to the $FabFc_2$.

These results may reflect differential binding of the two Abs to the various FcR. The PBMC-effector assay requires the presence of CD56-positive cells, presumably NK cells expressing FcγRIII, whereas the monocyte cytotoxicity assay is partially inhibited by Abs to each of FcγRI, FcγRII and FcγRIII (C. Hale, unpublished observations). These observations suggest that the humanized IgG1 may be more effective than the $FabFc_2$ construct in engaging FcγRI and/or FcγRII. Further experiments with blocking Abs to FcR may yield insight into these issues. Comparison with a FabFc construct may also be useful in this respect.

With regard to target cell interactions, the data presented in this report suggest that binding to the AT13/5 epitope is not strongly down-modulating, in agreement with previous reports for other anti-CD38 Abs (18). Under these conditions, one of the perceived advantages of the $FabFc_2$ construct is not realized. However, it has been argued that Ag modulation is more pronounced in vivo (74), and it may be that under these more complex conditions, the univalency of the $FabFc_2$ construct will become important.

Immunotherapy with anti-CD38

A central consideration in the design of any lytic immunotherapy is that of specificity. CD38 is by no means a tumor-specific Ag, in that it is widely expressed on cells of the hematopoietic system (75). During ontogeny, CD38 first appears on CD34-positive committed stem cells and lineage-committed progenitors of lymphoid, erythroid, and myeloid cells (76). CD38 expression persists only in the lymphoid lineage, through the early stages of T and B cell development. Mature resting lymphocytes generally express CD38 at low or undetectable levels. CD38 up-regulation is a marker for lymphocyte activation and particularly for B cell differentiation along the plasmacytoid pathway. Plasma cells are universally strongly positive for CD38 (16, 77).

Most importantly, however, for the use of anti-CD38 as an immunotherapeutic, the most primitive pluripotent stem cells of the hemopoietic system are CD38-negative (77, 78). Anti-CD38 immunotherapy would therefore be expected to have only transient effects on normal cells of the blood.

There is a solitary report of CD38 expression on mature nonlymphoid cells: a CD38-immunoreactive protein has been purified from E (79). The Ab described in this report does not label E in flow cytometry experiments (J. H. Ellis and K. A. Barber, unpublished observations).

Lytic anti-CD38 Abs have also therapeutic potential beyond the treatment of multiple myeloma. Plasma cells secreting self-reactive Ab underlie the pathology of conditions such as systemic lupus erythematosus and myesthenia gravis: these too should be lysed by the Abs described in this report. Indeed, the lack of restriction of CD38 expression to plasma cells widens the therapeutic relevance of anti-CD38 Abs. Other lymphoid tumors, most notably a subgroup of non-Hodgkins lymphomas, also exhibit a CD38-positive phenotype (80, 81), and presumably would be susceptible to anti-CD38-mediated cytotoxic attack. Also, as CD38 expression is up-regulated after activation of lymphocytes, this may offer a route to ablation of the self-reactive T lymphocytes found in rheumatoid arthritis and other non-Ab-mediated autoimmune diseases.

Finally, we note that despite the effectiveness of the humanized and $FabFc_2$ constructs in these simple in vitro tests, there is no assurance that their performance will be similar in the more complex in vivo environment of the myeloma patient. Although the data presented here provide no support for favoring a $FabFc_2$ construct over a conventional humanized IgG1, a direct comparison in vivo is required to provide a definitive assessment of immunotherapeutic potential. This issue is being addressed in ongoing clinical studies.

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Recognition of CD52 allelic gene products by CAMPATH-1H antibodies

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SUMMARY

Cloning of the CD52 from a B-lymphocyte tumour cDNA library revealed two closely related sequences differing only at two amino acids C-terminal to the proposed point of glycosyl-phosphatidylinositol (GPI)-linkage. When transfected into CHO cells only one of these sequences gave high-level expression of the antigen recognized by the prototypic anti-CD52 antibody CAMPATH-1 whereas in JURKAT cells good expression levels were obtained with both sequences. Fusion of the sequence from the second sequence to DNA encoding the extracellular domain of CD4 indicated that this sequence was capable of directing GPI linkage. The possible implications for the function of CD52 and serotherapy with anti-CD52 antibodies are discussed.

INTRODUCTION

The CD52 gene product is the antigen recognized by the CAMPATH-1 series of monoclonal antibodies.[1] This series of antibodies was identified by their ability to give very efficient lysis of human lymphocytes in the presence of human complement. Because of this property the CAMPATH-1 series has been developed for the serotherapy of lymphoid malignancies and autoimmune disease. CAMPATH-1 was the first antibody to be fully humanized and this humanized version (CAMPATH-1H) has been shown to have beneficial effects in non-Hodgkin's lymphoma[2] and rheumatoid arthritis.[3] The antigen recognized by the CAMPATH-1 series is CD52 and the structure of this molecule is now known in some detail. The mature peptide is a mere 12 amino acids in length and it is attached to the cell membrane via a glycosyl-phosphatidylinositol (GPI) linkage.[4,5] The polypeptide is *N*-linked glycosylated[6] explaining the apparent mobility on sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of 20 000–28 000 MW. The cDNA for the CD52 gene has been cloned and sequenced and shown to encode a 37-amino acid prepeptide which is subsequently processed to yield the mature CD52 peptide. It is postulated that the efficiency of anti-CD52 antibodies in lympholysis is partly due to the small size of the molecule and its lateral mobility in the membrane due to its GPI-anchorage. Both features which would allow antibody redirected complement or other effector mechanisms to be more effective.

In this study we report the successful expression of the CD52 in heterologous cells and confirm the presence of a second related sequence in human lymphocytes. Transfection of the second cDNA sequence gave much lower levels of expression of the CD52 antigen than the original sequence in CHO cells whereas in JURKAT cells the levels of expression were much more comparable suggesting differences in the efficiency of processing the gene product of the CD52 alleles in different cell types.

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MATERIALS AND METHODS

Cell culture

Wien 133 cells were cultured in filter-sterilized Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine and 500 IU/ml penicillin/500 μg/ml streptomycin. CHO dihydrofolate reductase (dhfr)$^-$ cells were detached by pretreatment with 0·02% versene containing 1·25% trypsin, washed in medium and cultured in filter sterilized Dulbecco's modified Eagles medium (DMEM) supplemented with 10% (v/v) heat-inactivated FCS, 2 mM L-glutamine, non-essential amino acids, hypoxanthine, thymidine and 500 IU/ml penicillin/500 μg/ml streptomycin. Transfected CHO dhfr$^-$ cells were cultured in DMEM supplemented with 10% (v/v) heat-inactivated *dialysed* FCS, 2 mM L-glutamine, non-essential amino acids and 500 IU/ml penicillin/500 μg/ml streptomycin. As the gene of interest became expressed, methotrexate, an inhibitor of dhfr, was added to the medium at an initial level of 3×10^{-8} M. This procedure selected for random amplification of the resident plasmid DNA associated with higher level gene expression. Once growth of the transfectants was stable at 3×10^{-8} M the methotrexate concentration was increased to 1×10^{-7} M. A repeat of several rounds of amplification led to maximal expression levels for the selected gene. The highest concentration of methotrexate employed was in the order of 1×10^{-6} M.

FACScan analysis and flow cytometry
Cell lines were washed and resuspended in fluorescence activated bell sorter (FACS) buffer (5% (v/v) FCS, 0·1% sodium azide (NaN_3) in phosphate-buffered saline (PBS)) at 1×10^5 cells/ml. The cells were dispensed into 96-well U-bottomed microtitre plates at 100 μl per well and 10 μl fluorescein isothiocyanate (FITC)-labelled antibody was then added. The cells were incubated at 4° for 30 min, to stain, before the plate was centrifuged at 1000 *g* in a Sorvall RT6000B bench centrifuge for 5 min. The excess liquid was removed and the cell pellets were resuspended in 100 μl wash buffer (0·1% sodium azide in PBS) and the process was repeated twice more. Once the cells had been fluorescently labelled they were finally resuspended in 100 μl FACS fix buffer (1% paraformaldehyde in PBS) and analysed on a FACScan (Becton Dickinson, Oxford, UK).

PIPLC treatment
Transfected CHO cells were diluted to 10^6/ml in medium and 100 μl either treated with 5 units of the enzyme phosphatidylinositol-specific phospholipase C (PIPLC, Boehringer Mannheim, Lewis, East Sussex, UK) or medium alone for 1 hr at 37°. The cells were then washed and stained with either FITC-labelled anti-CD4 or FITC-labelled CAMPATH-1H.

cDNA cloning and expression
To isolate the cDNA for the CD52 antigen from Wien 133, two 25-base pair (bp) polymerase chain reaction (PCR) primers were made which corresponded to either the 5′ sequences of the coding strand and which incorporated a *Hin*dIII site or to the 3′ sequences of the gene plus an included *Eco*RI site complementary to the coding strand, both based on the published sequence.[4] The Wien 133 cells were used to prepare total RNA by the guanadinium isothiocyanate method and the mRNA was isolated by magnetic separation on a Dynal column. 'SuperScript', (Gibco BRL, Paisley, UK), was used to produce a first strand cDNA and then a second strand copy was synthesized. A PCR performed on the resulting cDNA utilizing the primers mentioned previously produced an expected band of 206 bp. The band was purified on an agarose gel after cutting with the enzymes *Hin*dIII/*Eco*RI and the insert was cloned into either PUC 18 or PUC 19 cut with *Hin*dIII/*Eco*RI. After checking the sequence utilizing a Sequenase 2 kit (USB), the correct bands were excised from the holding vectors and cloned into an expression vector pRDN-1.

The vector, pRDN-1, contains the strong B-actin promoter and polyadenylation signals either side of a polylinker containing both *Hin*dIII and *Eco*RI sites therefore, it was possible to insert the CD52 antigen directly into the correct orientation. The vector also contains a dhfr gene cassette which permits possible selection and amplification of the correct antigen expressing colonies with methotrexate. After mapping positive colonies by restriction analysis the final selected plasmids pRDN AG and pRDN ΔC1H were grown up in large shake flasks and the plasmid DNA purified by caesium gradients. The CD52 containing DNA, was transfected into the CHO dhfr$^-$ line B11 using Promega Transfectam. Successful transfection with the CD52 constructs was monitored by methotrexate resistance and surface expression of the antigen, as assessed by FACS analysis, with a wide spread of expression levels seen only for the authentic CAMPATH-1 construct. Following amplification of both constructs in 3×10^{-8} M methotrexate, single highly expressing or resistant clones of the transfected cell lines were isolated by dilution cloning.

CD4/CAMPATH antigen chimeric constructs
Full length extracellular CD4 was jointed to the GPI-displaced signal peptide of either the construct pRDN AG or pRDN ΔC1H via PCR utilizing overlap primers designed to correspond to either the proline residue at residue +373 in CD4 or the proline residue +11 in CAMPATH antigen CDw52 whilst retaining the original frame. The resulting constructs named pRDN CD4AG and pRDN CD4ΔC1H were confirmed as correct via sequencing with the Sequenase 2 kit supplied by USB. Both constructs, as well as full length CD4 or CAMPATH antigen in pRDN-1, were transfected into CHO dhfr$^-$ cells as before and amplified to 3×10^{-8} M methotrexate prior to dilution cloning.

RNAase protection assay
CHO cells containing either pRDN AG, pRDN ΔC1H or the parental cells were used as a source of template for RNAase protection assays. A certain number of cells, i.e. 10^7/ml or 10^6/ml, were lysed with the lysis buffer from a USB RNA protection kit and the lysates were mixed with either single-stranded forward or reverse ^{32}P-labelled probes made by RNA runoffs from the whole CAMPATH antigen cloned into a Bluescript vector. The reactions were left overnight at 37° before the products were treated with RNAase to remove excess probe and the products separated on a 6% sequencing gel. The dried gel was autoradiographed.

In vivo *experiments*
Parental or transfected CHO cells to be studied were harvested from flasks, washed and resuspended in PBS at 2×10^7/ml before being mixed with an equal volume of 6 mg/ml matrigel for injection subcutaneously (0·1 ml) into nude mice. After 1 month, approximately, tumours were excised from the animals, teased into single cell suspensions and either analysed by FACS for CD52 expression.

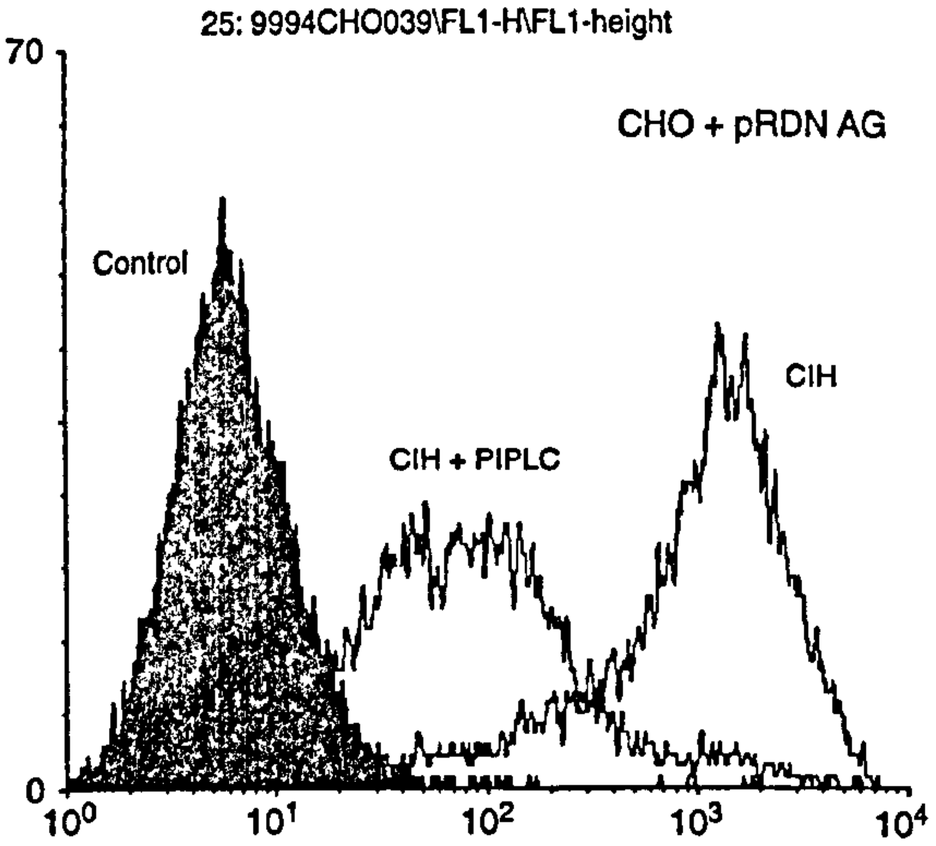
Immunoprecipitations and Western blotting
CHO cells were lysed in 3% nonidet P-40 (NP-40) lysis buffer (containing 20 mM MOPS, 15 mM EGTA, 3% NP-40, 2 mM phenylmethylsulphonyl fluoride, 1 mM Na_2VO_4 (10 μg/ml aprotonin, 2 mM EDTA) for 15 min on ice. After which time the lysate was centrifuged at 10 000 *g* for 5 min at 4°. The clarified supernatant was precleared with Sepharose-4B for 30 min at 4°, and the precleared lysate incubated with Q4120 (anti-CD4, Sigma, St Louis, MO)–coupled Sepharose for 1 hr at 4° with end-over-end rotation. The beads were then washed twice in lysis buffer and resuspended in non-reducing SDS–PAGE sample buffer and boiled for 3 min. The samples were run on 8% SDS–PAGE gels, blotted onto nitrocellulose and the blots blocked with 3% non-fat milk in PBS. The blots were then probed with biotin-Q4120 followed by streptavidin–horseradish peroxidase and developed by enhanced chemiluminescence (ECL, Amersham, Amersham, UK).

RESULTS

Cloning and expression of the CD52 gene

Complementary DNA was prepared from the B-cell lymphoma Wien 133 and used as a template for the PCR amplification

of a 206-bp fragment containing the cDNA for CD52. A cDNA clone corresponding to the reported sequence for CD52[4] was cloned into the mammalian expression vector pRDN-1 and the resulting construct transfected into both dhfr/CHO cells and JURKAT T cells. In the case of CHO cells there was no requirement for co-transfection with plasmid containing a selective marker as pRDN-1 contains the dhfr gene. JURKAT were co-transfected with the CD52 gene in pRDN-1 and the p321 plasmid which confers neomycin resistance. Both cell lines expressed the CD52 gene product as recognized by the anti-CD52 antibody CAMPATH-1H (Fig. 1), indicating that there are apparently no stringent species or cell lineage requirements for the expression of this human leucocyte antigen. Untransfected CHO cells did not stain with CAMPATH-1H (data not shown and Fig. 2). Parental JURKAT cells have low to negligible levels of CD52, Fig. 1 shows three subclones of JURKAT cells expressing different levels of CD52 after transfection, indicating that there is some clonal variation in the expression of CD52, analysis of pooled transfectants was subsequently used to assess efficiency of expression of CD52. The expression of CD52 on CHO cells was reduced by treatment with PIPLC indicating that, as in lymphocytes, the CD52 antigen is linked to the membrane via a GPI-anchor (Fig. 1).



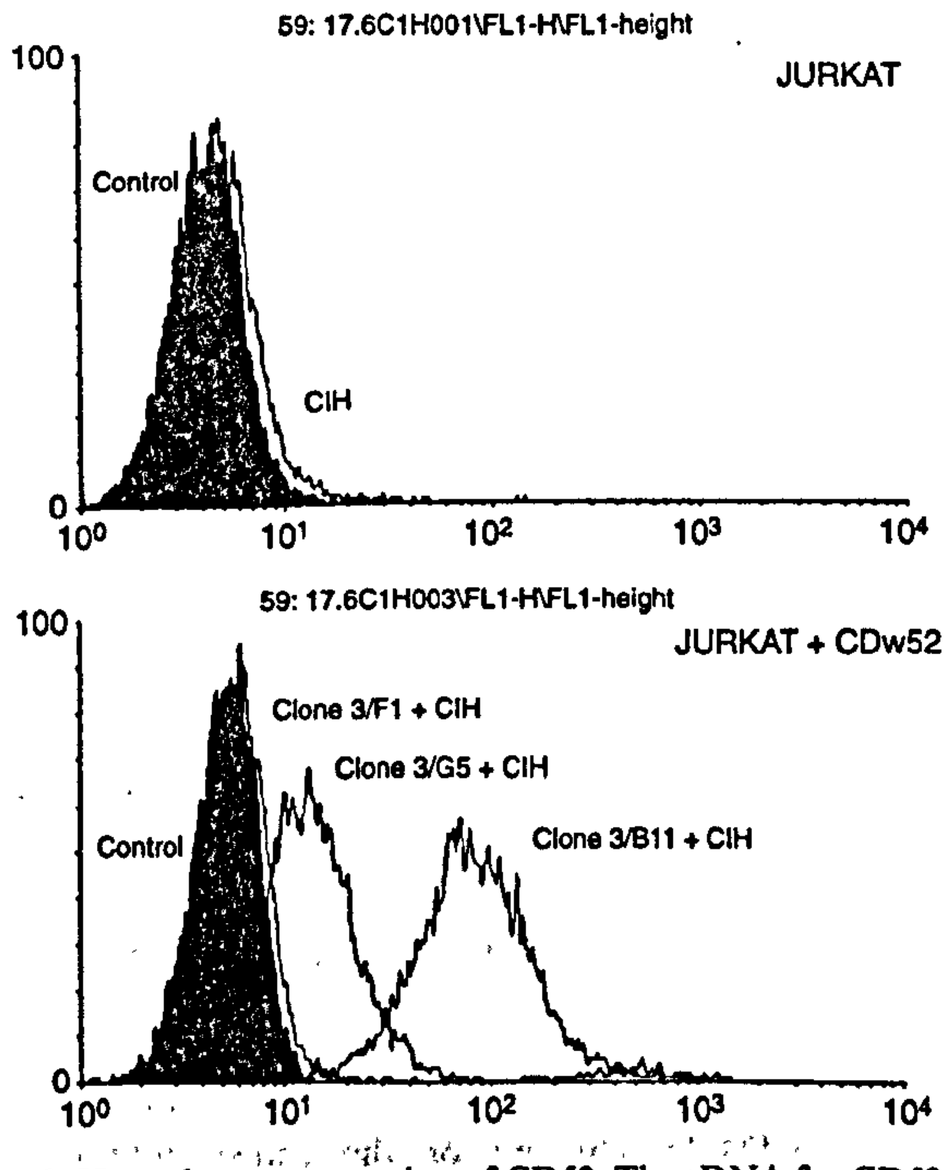


Figure 1. Heterologous expression of CD52. The cDNA for CD52 was transfected into either CHO cells or JURKAT cells. After appropriate selection, cells were stained with FITC-labelled CAMPATH-1H and analysed by flow cytometry. Treatment with PIPLC to remove GPI-anchored proteins was performed as described in the Materials and Methods. (JURKAT, control JURKAT cells; JURKAT + CDw52, JURKAT cells transfected with cDNA encoding CD52).

Sequence analysis of the CD52 gene

During the cloning and sequencing of the PCR products from the Wien 133 cDNA it became apparent that two sequences were represented in the cDNA. These were found in approximately equal proportions amongst independent clones (Table 1). The two sequences were AAC ATA and AGC ATG at positions 151–156 which would result in changes from Asn-Ile to Ser-Met at positions 16 and 17 of the mature peptide, a site

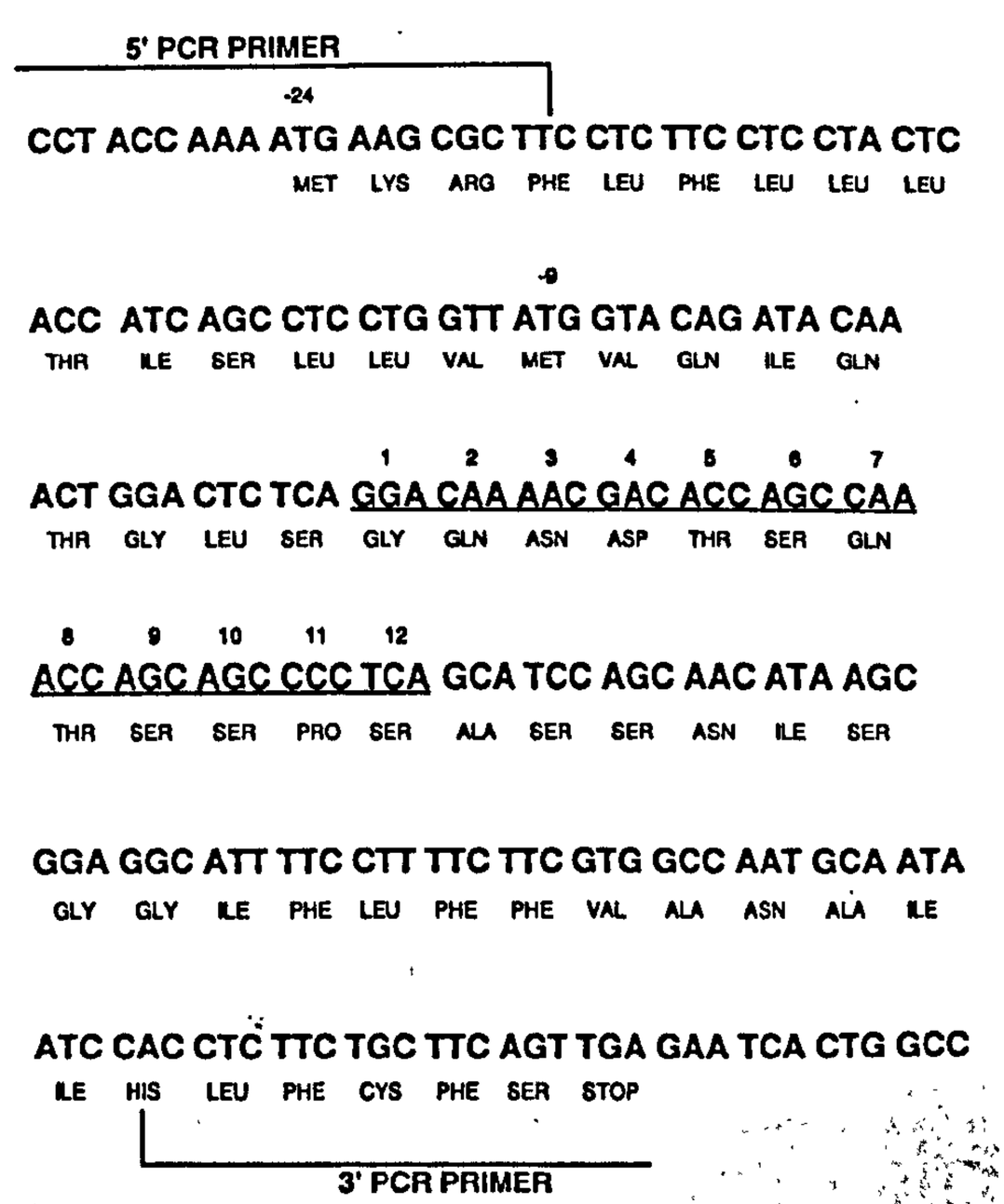


Figure 2. The DNA sequence of the CD52 gene is shown with the primers use for the PCR reactions to amplify the gene.

Table 1. Sequence analysis of PCR inserts

	CDw52	ΔC1H
Exp. 1	1/2*	1/2
Exp. 2	4/8	4/8
Exp. 3	2/3	1/3
Total	7/13	6/13

*Data are expressed as the number with that particular sequence divided by the total number sequenced for each experiment.

on the C-terminal side of the proposed GPI-anchorage site (Fig. 2). This difference was also noted by Xia *et al.*[4] in independently isolated cDNA clones but since only one clone was isolated the authors were unable to conclude whether this sequence represented a second allele or a cloning artefact. Since the observed changes were in the region of the site for the addition of the GPI-anchor (Fig. 3) we decided to clone and express this second variant. Figure 4 shows the result of stable transfection of the two forms into CHO cells. The original version (CD52) led to high-level expression of CD52 as measured by CAMPATH-1H staining. The second form ΔC1H showed only a very low level of expression of the CD52 antigen. Ribonuclease protection assay analysis indicated that both transfectants were making the appropriate mRNA (Fig. 5). The difference in expression levels was maintained throughout the amplification with methotrexate (data not shown). The epitope recognized by the CAMPATH-1H antibody is composed of contributions from both the peptide sequence and the GPI-anchor.[5] Thus it is possible that if the observed change in peptide sequence leads to a change in the position of the attachment of GPI-anchor then the antigen expressed from the alternative sequence will not be recognized by the CAMPATH-1H antibody. In order to test whether the alternative sequence could function as a membrane-anchoring

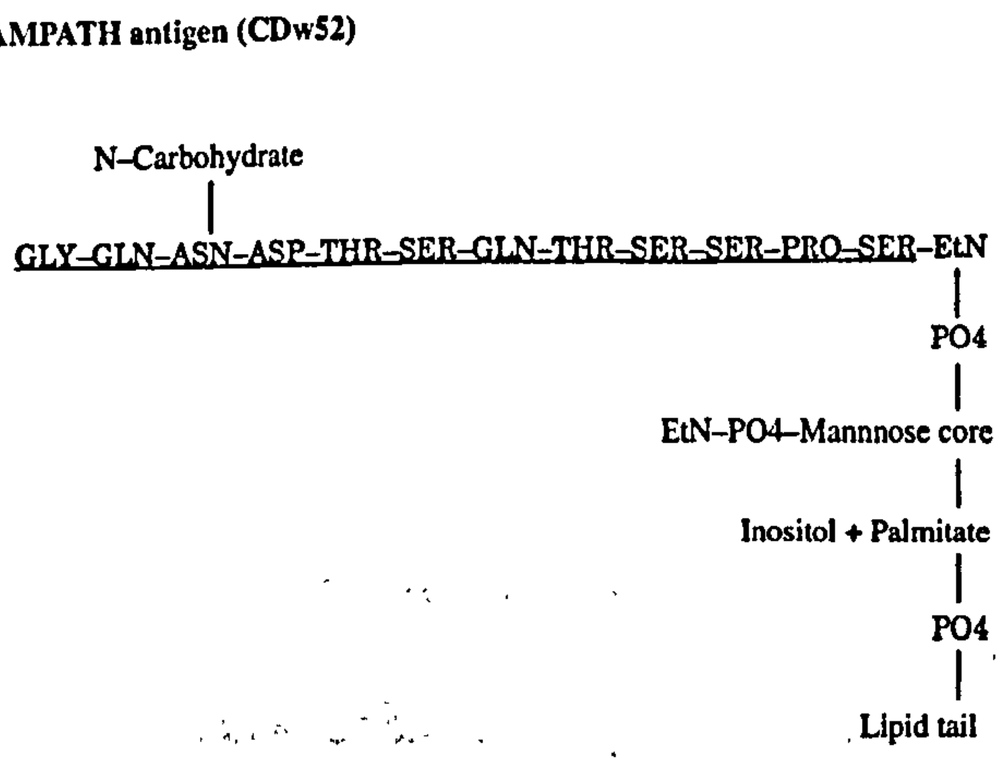


Figure 3. The amino acid sequence of the CD52 gene product with the proposed site of the GPI-anchor is shown.

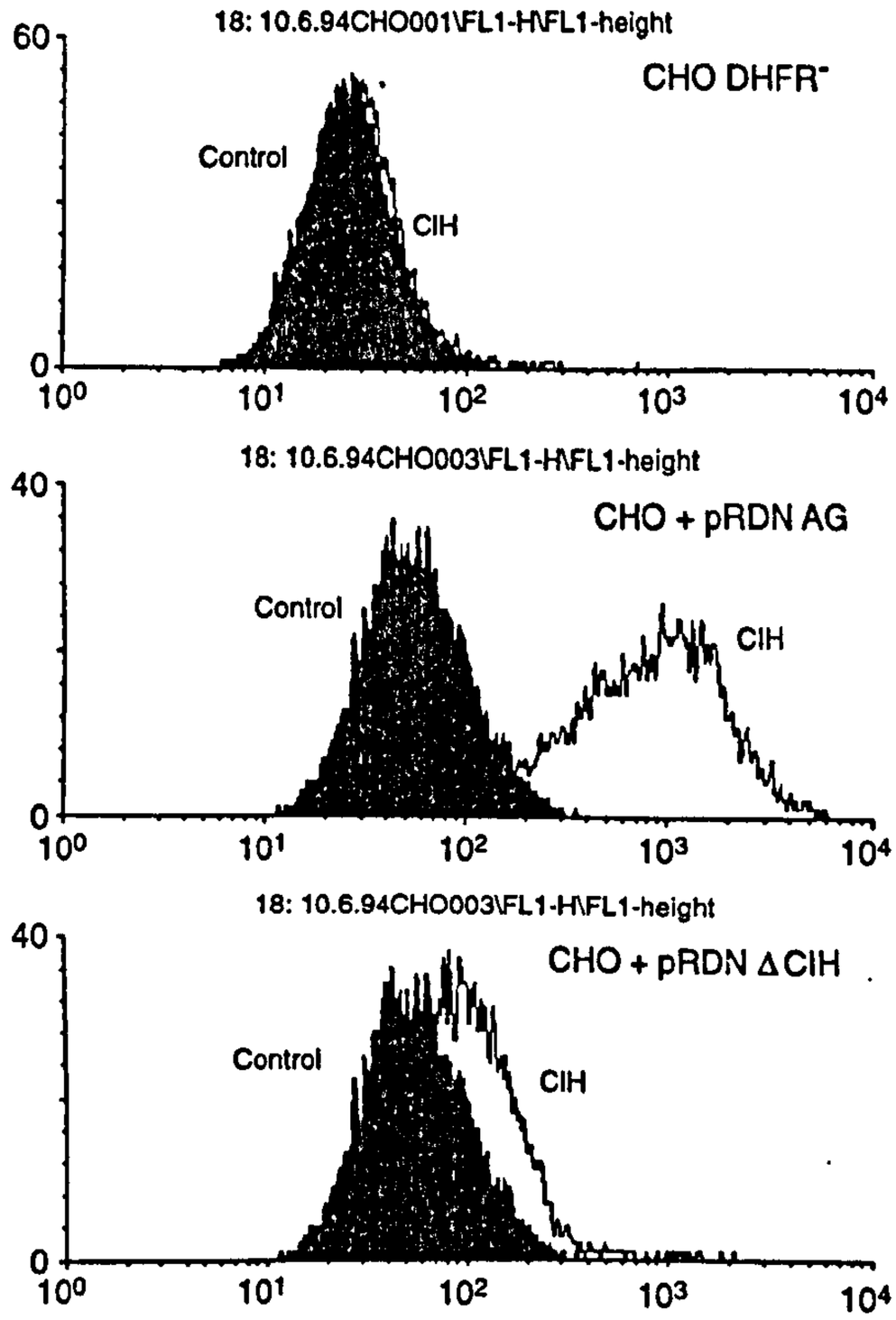


Figure 4. Expression of the two related CD52 genes; cDNA for CD52 (pRDN AG) or the alternative sequence pRDN ΔC1H were transfected into CHO cells and appropriate selection and amplification applied. Untransfected CHO cells (CHO DHFR⁻) pRDN AG-transfected CHO cells (CHO + pRDN AG), or pRDN ΔC1H-transfected CHO cells were stained with FITC-labelled CAMPATH-1H and analysed by flow cytometry.

sequence it was therefore necessary to attach another determinant to the potential anchor sequences.

Construction of CD4 extracellular domain–CD52 chimerae

In order to determine whether the CD52 sequence could function as a membrane-anchoring sequence, chimeric genes were constructed consisting of the coding regions for extracellular domains of CD4 and the anchoring sequence from CD52 or ΔCIH. These constructs were transfected into CHO cells and monitored for CD4 expression. Both chimeric molecules were expressed as detected by staining with anti-CD4 antibody (Fig. 6). The replacement of the extracellular domain of CD52 with that of CD4 prevented the detection with anti-CD52 antibodies. In fact, transfectants expressing the CD4/ΔCIH chimera showed higher levels of CD4 staining than either the CD4/Ag chimera or the transmembrane CD4 construct. Furthermore, immunoprecipitation analysis indicated that the transfected constructs yielded recombinant proteins of the correct size, those constructs encoding GPI-anchored molecules being smaller than the full-length CD4 molecule (Fig. 7). Thus both structures can direct the extracellular domain of CD4 to the cell membrane. The

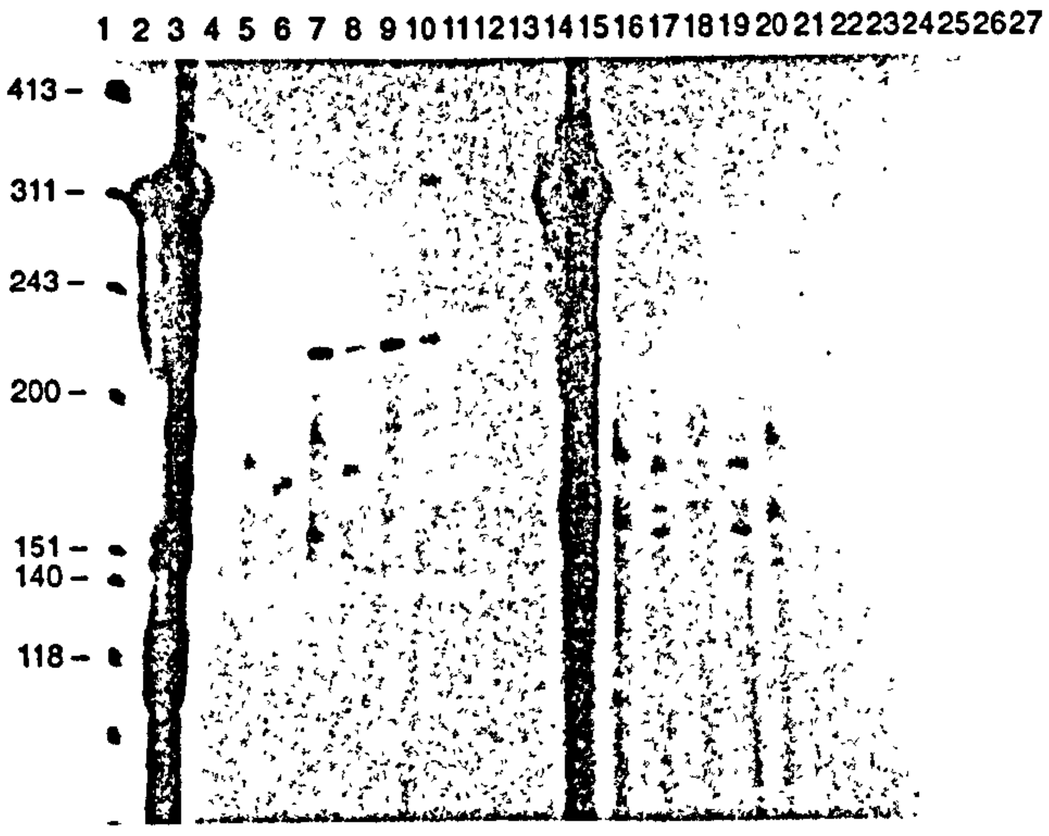


Figure 5. Measurement of mRNA levels by RNAase protection; CHO cells transfected with pRDN AG or CHO cells transfected with pRDN ΔC1H were used as template for RNA protection studies. Either 10^7 cells/ml or 10^6 cells/ml were incubated with CAMPATH antigen probes transcribed in forward or reverse orientation in the Bluescript vector KS$^+$ (10^5 c.p.m. per reaction). Lane 1, ^{32}P end-labelled Ø174 marker cut with *Hinf*; lane 3, 5 μl reverse probe; lanes 5–10, cell lysates plus reverse probe digested with RNAase of which lane 5 10^7/ml CHO cells, lane 6 10^6/ml CHO cells, lane 7 10^7/ml CHO cells plus pRDN AG, lane 8 10^6/ml CHO cells with pRDN AG, lane 9 10^7/ml CHO cells with pRDN ΔC1H, lane 10 10^6/ml CHO cells with pRDN ΔC1H; lanes 2, 4, 11–13 blank; lane 14, 5 μl forward probe; lanes 15–20, cell lysates plus forward probe digested with RNAase of which lane 15 10^7/ml CHO cells, lane 16 10^6/ml CHO cells, lane 17 10^7/ml CHO cells plus pRDN AG, lane 18 10^6/ml CHO cells with pRDN AG, lane 19 10^7/ml CHO cells with pRDN ΔC1H, lane 20 10^6/ml CHO cells with pRDN ΔC1H; lanes 21–27 blank. Lanes 7–10 show the expected band at 206 bp.

transfectants were treated with PIPLC to determine whether the CD4 was linked via a GPI-anchor, in both cases expression levels were diminished by this treatment. By contrast CHO cells transfected with a construct encoding a full-length CD4 cDNA containing a transmembrane and cytoplasmic domain expressed CD4 which was unaffected by PIPLC treatment (Fig. 6).

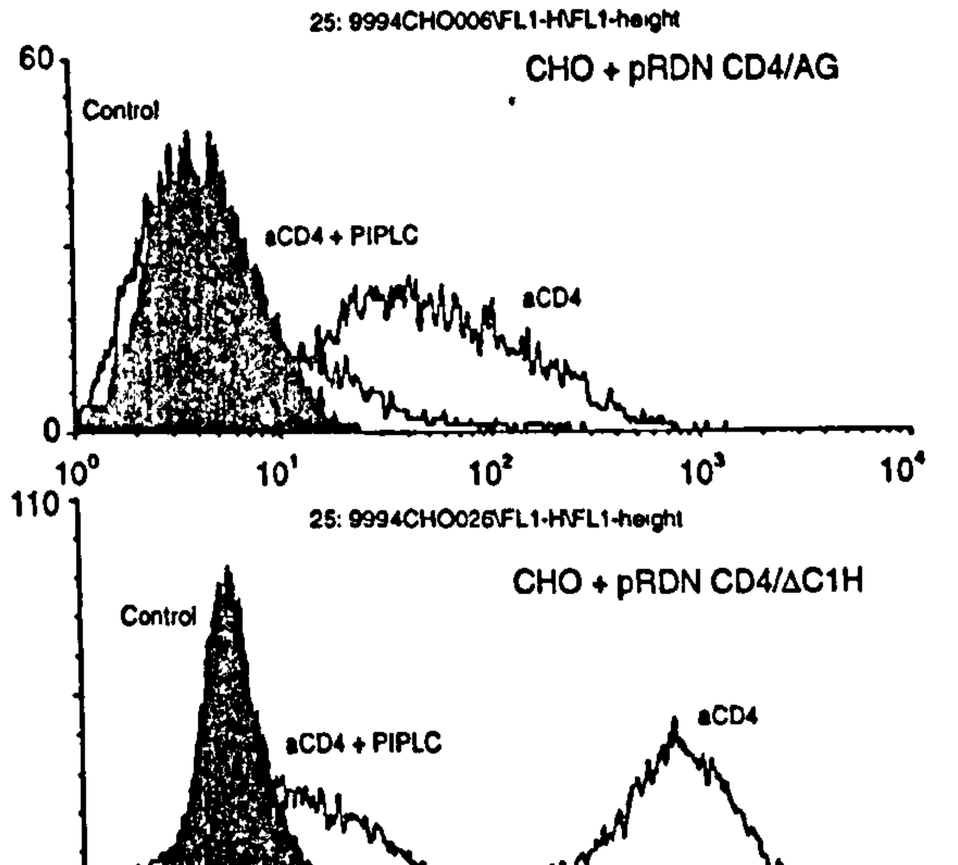


Figure 6. PIPLC treatment of transfectants; pRDN CD4/Ag, pRDN CD4/ΔC1H and CD4 CHO cell transfectants were incubated with either medium alone or PIPLC and then stained with FITC-labelled anti-CD4 and analysed by flow cytometry.

Expression of CD52 alleles in JURKAT cells and normal human peripheral blood mononuclear cells

In order to determine whether the inefficient expression of the alternative CD52 allele in CHO cells was related to cell type, the cDNA for both alleles were transfected into JURKAT T cells and the transfected pools were subjected to flow cytometric analysis. Figure 8 shows that both alleles are efficiently expressed in this lymphocytic cell line. However, even in this cell line the expression of the alternative allele is somewhat lower than the originally described allele, possibly reinforcing the notion that this gene product is less efficiently processed, the originally described allele showing an approximately sevenfold higher peak fluorescence than the alternative allele. The expression of the two alleles in normal peripheral blood mononuclear cells (PBMC) was investigated by sequencing the PCR products amplified from cDNA prepared from 11 normal individuals. In two of 11 individuals only the originally described allele could be found, in six the alternative allele was the only product present and in the other three both products could be isolated.

Flow cytometric analysis of the PBMC indicated that all the individuals expressed CD52 at approximately the same level (data not shown).

Morphological and functional characteristics of transfectants

The function of the CD52 gene product is currently unknown. Other GPI-anchored structures such as CD59[7] and CD55[8] have been shown to act to protect cells from complement attack. During the course of experiments in which we expressed the two CDw52 sequences we noticed a major difference in morphology between the two transfected cell lines. Whereas untransfected CHO cells and CHO cells transfected with the ΔCIH gene product were rounded in appearance, cells transfected with the originally described CD52 gene adopted an elongated, spindly morphology (Fig. 9). We wished to determine whether this difference may have any corollary in terms of functional behaviour. To determine whether expression of the different

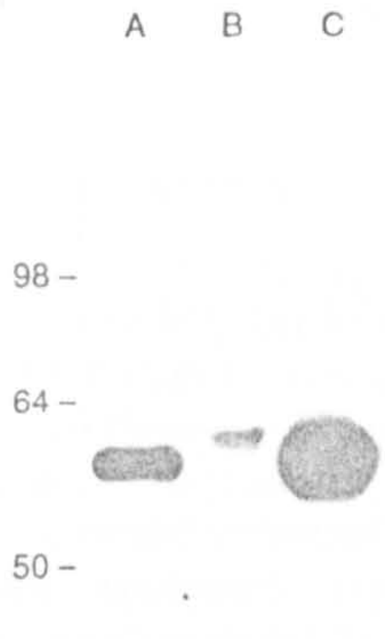


Figure 7. Immunoprecipitation analysis of transfectants; Lysates from CHO pRDN CD4/AG, CHO pRDN CD4/ΔC1H, CHO-CD4 or CHO cells were immunoprecipitated using anti-CD4 Sepharose and the immunoprecipitated proteins were separated by SDS–PAGE, transferred to nitrocellulose by Western blotting and probed with anti-CD4 antibodies. Lane A, pRDN CD4/AG; lane B, pRDN CD4/ΔC1H; lane C, CHO-CD4; lane D, CHO.

genes had an effect on growth *in vivo*, 1×10^6 cells of each cell type were transplanted subcutaneously into nude mice and monitored for tumour growth. Table 2 shows the data from two separate experiments in which the identical results were obtained. Only mice receiving CHO cells expressing the CD52

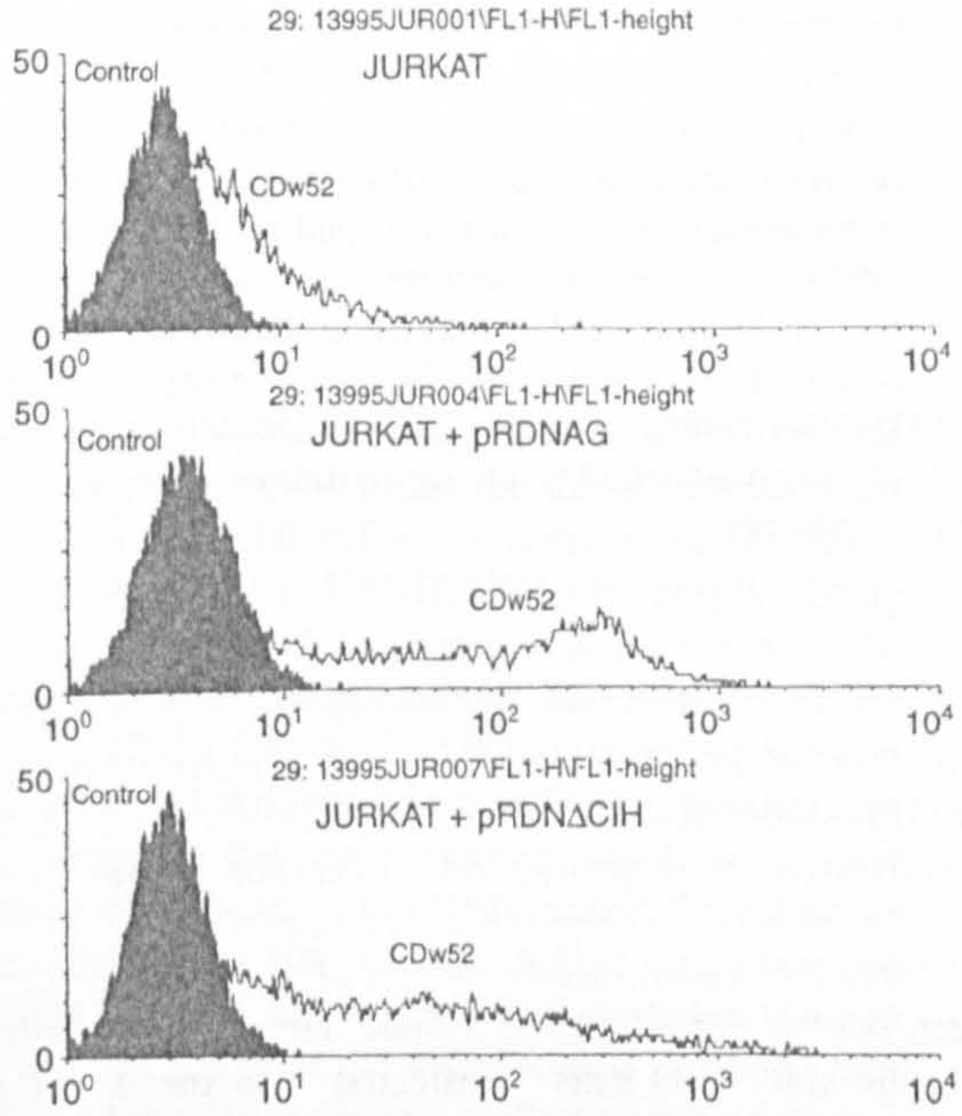


Figure 8. Expression of CD52 alleles in Jurkat T cells; Jurkat T cells were transfected with either pRDN AG or pRDN ΔC1H as described in the Materials and Methods. The transfected T cells were stained after passage in select medium with FITC-labelled CAMPATH-1H antibody as indicated.

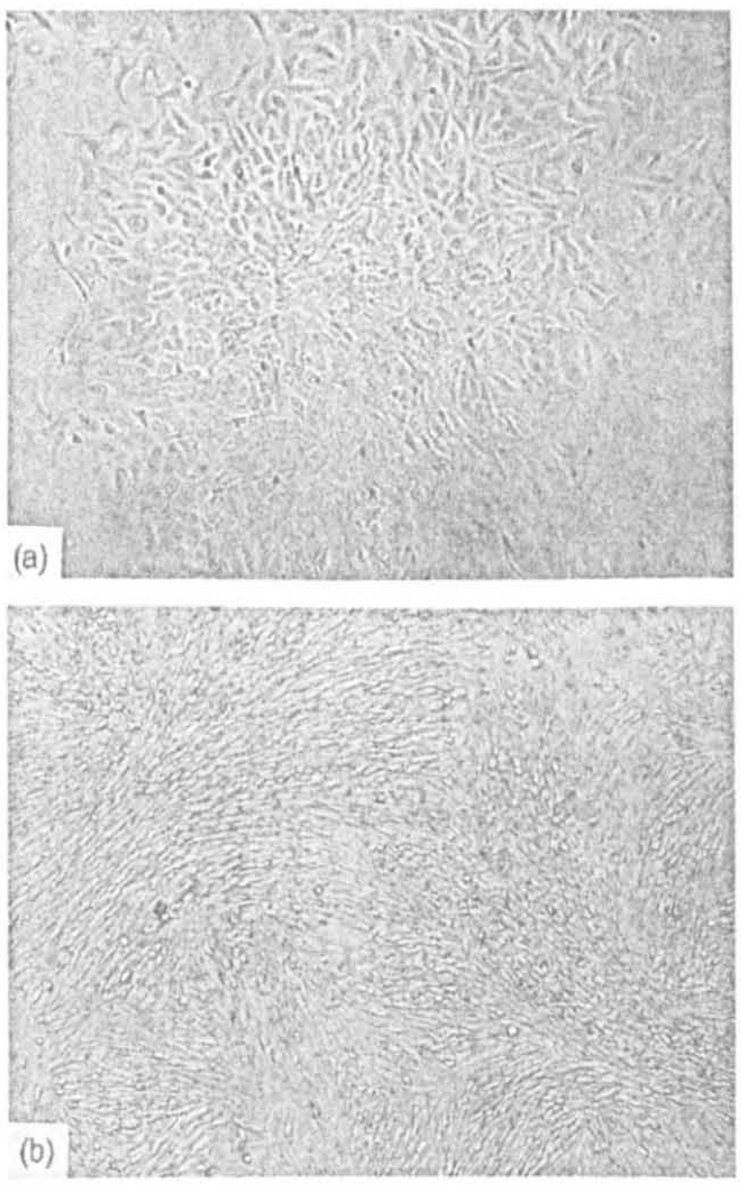


Figure 9. Morphological differences between transfected cell lines photomicrographs of subconfluent CHO cells. (a) Parental CHO cells (b) pRDN AG-transfected CHO cells, and (c) pRDN ΔC1H transfected cells.

(CHO/pRDN AG) gene presented with tumours, mice receiving untransfected CHO cells or CHO cells transfected with the ΔCIH gene (CHO/pRDN ΔCIH) showed no sign of tumour growth. These data may indicate that the CD52 gene product may play a role in either cell–cell adhesion or protection of the

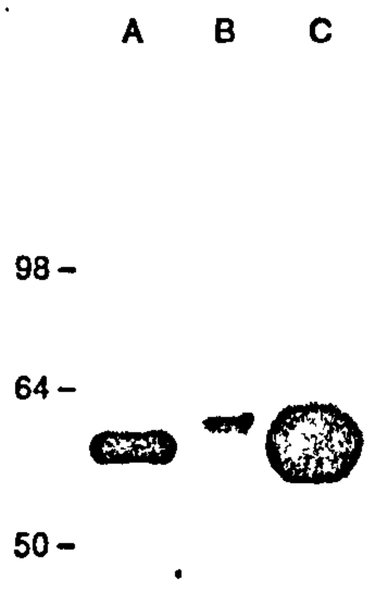
Table 2. Growth of CHO transfectants *in vivo*

	CHO	CHO/pRDN AG	CHO/pRDN ΔC1H
Exp. 1	0/10*	4/10	0/10
Exp. 2	0/10	3/10	0/10
Exp. 3	0/10	9/10	ND†
Total	0/30	16/30‡	0/20

* Data are expressed as the number of mice exhibiting tumour growth divided by the total number of mice inoculated.

† ND, not determined.

‡ $P < 0{\cdot}001$ according to χ^2 analysis.



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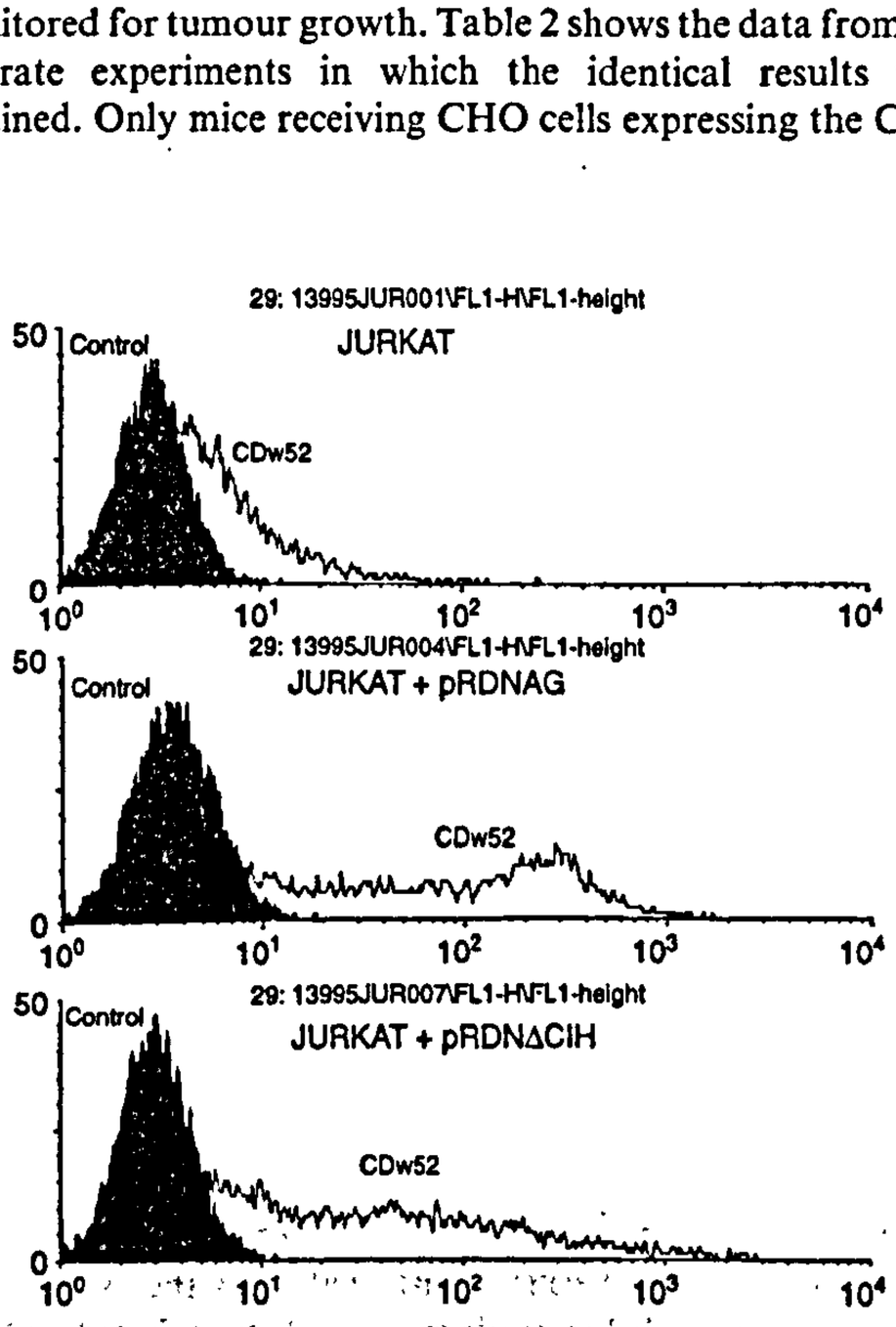

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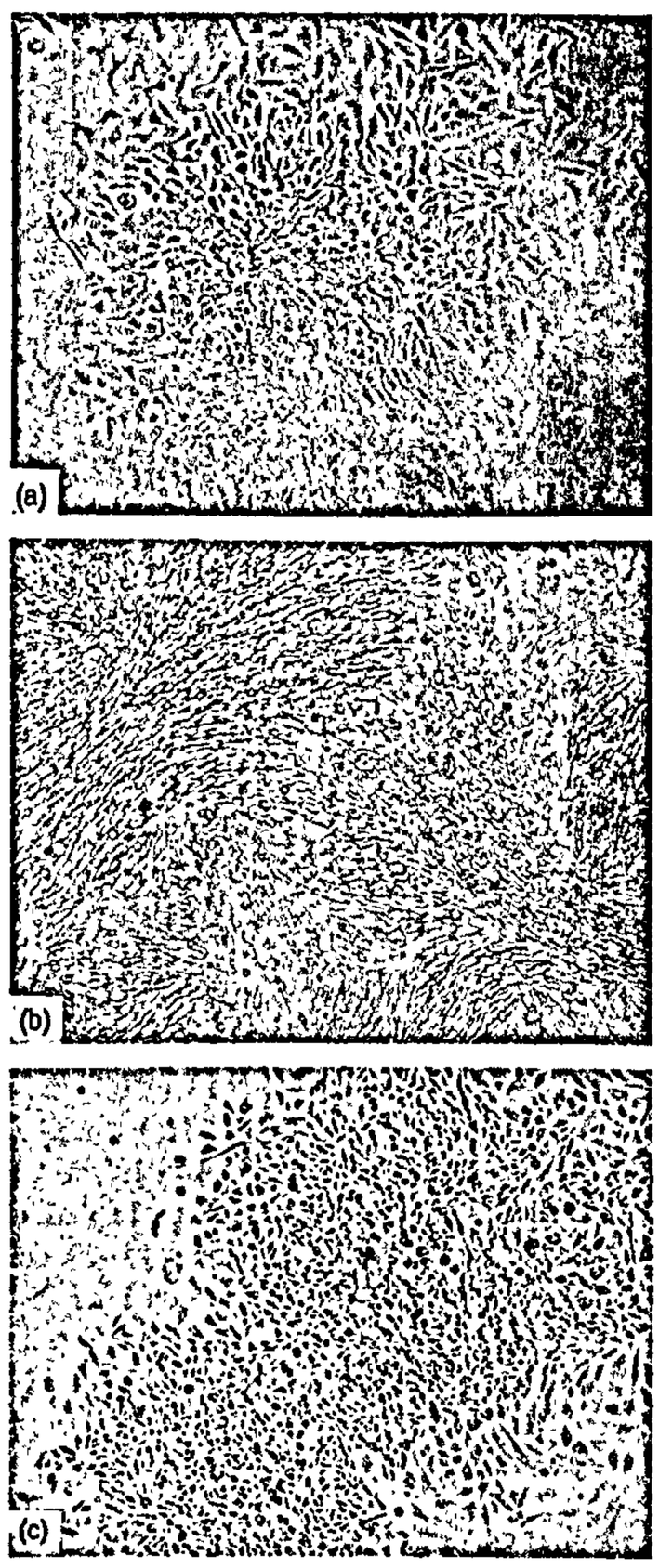

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Exp. 3	0/10	9/10	ND†
Total	0/30	16/30‡	0/20

*Data are expressed as the number of mice exhibiting tumour growth divided by the total number of mice inoculated.

†ND, not determined.

‡ $P < 0.001$ according to χ^2 analysis.

cell from extracellular influences, both roles would be consistent with enhanced survival of the transfectants *in vivo*.

DISCUSSION

The CD52 antigen was first discovered by screening for antibodies which were effective in fixing human complement.[1] Rat antibodies of the CAMPATH-1 series were the prototype anti-CD52 antibodies and these have been developed for therapeutic use as lymphocyte-depleting agents in the treatment of lymphoid malignancy and autoimmune disease.[2,3] The interest in the potential therapeutic exploitation of the CAMPATH-1 series has been paralleled by research into the nature of the antigen. Xia *et al.* showed that the CD52 antigen is a 12-amino acid-glycosylated polypeptide which is linked to the plasma membrane via GPI-linkage.[5] The determinant recognized by CAMPATH-1 is formed from a contribution from both the peptide backbone and the GPI-anchoring. The requirements for the attachment of a GPI-anchor are to have a small amino acid such as serine—to which the anchor is attached—separated by 10–12 amino acids from a strongly hydrophobic domain.[9,10]

During the cDNA cloning of CD52 Xia *et al.*[4] noted that two closely related sequences were found, differing at two amino acids C-terminal to the GPI-anchoring site. Whereas the CD52 cDNA codes for Asn-Ile in these two positions, the alternative ΔCIH encodes Ser-Met. When transfected into CHO cells the 'wild-type' version of the gene encoding Asn-Ile at positions 16 and 17 led to high-level expression of the antigen recognized by the CAMPATH-1H antibody. Subsequently there has been an independent report in which the two sequences have been noted.[11] We first expressed CD52 in CHO and JURKAT cells and were able to detect the antigen with CAMPATH-1H antibody. The antigen was sensitive to treatment with PIPLC indicating a GPI-linkage to the cell membrane. The expression in both hamster and human cells of differing tissue origin suggests that there is no tissue or species specificity controlling the expression of the originally described CD52 allele. This is similar to other GPI-anchored proteins such as CD55 and CD59 which have also been expressed in heterologous cell lines such as CHO or Hela cells.[12,13] CHO cells transfected with the alternative Ser-Met version of the gene expressed much lower levels of CD52 despite an identical sequence in the coding region for the predicted CD52 peptide backbone. Two possible explanations for this observation are first, that the GPI-linkage differs for this sequence and forms an antigen not recognized by CAMPATH-1H despite being expressed, or second, that the alternative sequence is inefficiently processed in these transfectants. The analysis of the processing of the CD52 GPI-anchor is hampered by the fact that there are a limited number of anti-CD52 antibodies and the majority recognize the same epitope which is at least partially made up of elements of the GPI-anchor. Therefore, to study further the properties of the CD52 sequence we constructed CD4/CD52 chimeric genes and expressed these in CHO cells. Flow cytometry of the transfectants indicated that both the CD52 and the ΔCIH sequences allowed the membrane expression of CD4 and furthermore in both cases the expression was sensitive to treatment with PIPLC, CHO cells expressing full-length transmembrane-anchored CD4 were also treated with PIPLC and in this case CD4 expression was found to be PIPLC-insensitive. Thus, the ΔCIH sequence can be processed to generate a GPI-anchor with the CD4 extracellular domains, in fact expression levels are somewhat higher than with the original CD52 sequence. It is therefore possible that the inability to obtain CAMPATH-1H reactivity with ΔCIH is due to an inefficiency of the entire ΔCIH sequence to be processed and transported to the cell surface. This is not however due to an intrinsic inability of the ΔCIH sequence to form a GPI-linkage. Other possibilities are that the ΔCIH gene product is processed but that the product is not recognized by antibodies of the CAMPATH-1 series possibly due to the attachment of the GPI-anchor at an alternative site or possibly due to more rapid turnover or degradation.

The existence of two forms of the CD52 gene has now been reported by several independent laboratories.[4,11] If the CD52 gene has different alleles it is interesting to speculate what effect this would have on expression of CD52. In the normal population there have been no reports of $CD52^-$ patients suggesting that if people homozygous for the alternative form exist they express the antigen efficiently. Indeed our analysis of PBMC from normal donors indicates that a significant proportion have only the second allele and yet express high levels of CD52 on their lymphocytes. This is consistent with our data obtained with CD4–CD52 chimeric molecules but not with the low-level expression of CD52 in CHO cells transfected with the alternative sequence. A possible explanation is that the alternative gene product is less efficiently processed in CHO cells leading to low expression levels. In lymphocytes the gene may be processed more efficiently leading to higher levels of expression, consistent with this possibility is the fact that the expression of CD52 on normal lymphocytes is very high. The experiment shown in Fig. 8 supports this hypothesis. Both alleles are efficiently expressed in JURKAT T cells, however even in the T-cell line there is better expression in the transfected cell pool with the original allele, with almost a sevenfold difference in the peak fluorescence. This compares to an approximate tenfold difference in the CHO cell transfectants.

The function of the CD52 gene product is not known at present. The data presented here suggest that expression of CD52 in CHO cells gives them a selective growth advantage *in vivo*. Interestingly, the pRDN ΔCIH transfectants were similar to CHO cells both morphologically and in terms of *in vivo* tumorigenicity. This suggests that the relatively inefficient expression of CD52 by these cells does not lead to the changes in morphology and the same selective advantage. Such an observation would be consistent with either a role for CD52 in cell protection from the host environment or with a role in cell–cell adhesion both of which would favour enhanced survival. The transfected cell lines described here may prove to be extremely useful reagents for the elucidation of the function of CD52.

ACKNOWLEDGMENT

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Glycosylation and biological activity of CAMPATH-1H expressed in different cell lines and grown under different culture conditions

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CAMPATH-1H (where CAMPATH is a trade mark of Wellcome group companies), a humanized IgG antibody used in the therapy of lymphoma, leukaemia and rheumatoid arthritis, has been expressed in Chinese hamster ovary, Y0 myeloma and NS0 myeloma cell lines. These engineered cell lines were grown under different culture conditions, and the antibody isolated and purified. N-Linked oligosaccharides, on the CH_2 heavy chain region of the antibody, were isolated and analysed by hydrazinolysis, high-performance anion-exchange chromatography with pulsed amperometric detection, laser-desorption mass spectrometry and sequential exoglycosidase treatment. Both the glycosylation pattern and the biological activity of CAMPATH-1H, as measured by antibody-dependent cell-mediated cytotoxicity, were markedly affected by the cell line used to express the antibody. It is concluded that glycosylation of the antibody may be important in the clinical outcome of therapy.

Key words: ADCC/antibody/CAMPATH/glycosylation/oligosaccharide

Introduction

Glycosylation of therapeutic antibodies can have a critical effect on biological activity and pharmacokinetics (Nose and Wigzell, 1983; Parekh *et al.*, 1985; Wright *et al.*, 1991). The removal of the N-linked oligosaccharide from the CH_2 heavy chain region of the antibody abrogates target cell killing by complement-mediated lysis (CML) and antibody-dependent cell-mediated cytotoxicity (ADCC) (Nose and Wigzell, 1983; Tao and Morrison, 1989). Circulatory lifetime, immunogenicity and antigenicity of the antibody may also be affected by glycosylation of the molecule (Rademacher *et al.*, 1988). There are several factors that can influence glycosylation. The species, tissue and cell type have all been shown to be important in the way that glycosylation occurs (Rademacher *et al.*, 1988). In addition, the extracellular environment, through altered culture conditions such as serum concentration, may have a direct effect on glycosylation (Goochee and Monica, 1990; Maiorella *et al.*, 1993).

CAMPATH-1H (where CAMPATH is a trade mark of Wellcome group companies), a humanized IgG1 antibody (Riechmann *et al.*, 1988), is directed against the CD52 antigen (Hale *et al.*, 1990; Treumann *et al.*, 1995) present on the cell surface of the majority of normal and malignant lymphocytes. This antibody has been expressed in several mammalian cell lines, including Y0 rat myeloma, NS0 mouse myeloma and Chinese hamster ovary (CHO), grown using different culture conditions, and extensively tested in clinical trials of lymphoproliferative disorders (Hale *et al.*, 1988) and rheumatoid arthritis (Isaacs *et al.*, 1992), with some major successes.

This study was initiated in order to determine whether differences in glycosylation in CAMPATH-1H were apparent between the different cell lines expressing the antibody, and whether there were corresponding differences in *in vitro* biological activity, the natural consequence being that glycosylation of the antibody may be important in the clinical outcome of therapy.

Results

Purification and chemical characterization of CAMPATH-1H

CAMPATH-1H was purified from culture supernatants by protein A–Sepharose chromatography. SDS–PAGE (data not shown) showed a single band at 150 kDa (non-reducing gel), and bands at 50 and 25 kDa (reducing gel) for heavy and light chains, respectively. No evidence for free heavy or light chain was observed. Size-exclusion HPLC indicated that the material was present as the monomer with <1% aggregate.

Preparation and analysis of oligosaccharide standards

A series of oligosaccharide standards was obtained commercially, and further standards were derived from these by treatment with exoglycosidases, using β-galactosidase, α-fucosidase and/or β-*N*-acetylhexosaminidase. Table I shows the relative retention times of these oligosaccharide standards, analysed by high-performance anion-exchange chromatography (HPAEC), together with the corresponding [M + Na^+] molecular ions observed by laser-desorption mass spectrometry (LD-MS). All structures were as expected and fully confirmed by LD-MS. It is clear that removal of galactose (Gal) and/or *N*-acetylglucosamine (GlcNAc) from the outer arm of the oligosaccharide reduces, whereas removal of the core fucose (Fuc) residue increases, the relative retention time on HPAEC.

N-Linked oligosaccharides from CAMPATH-1H

Oligosaccharides were released from CHO, Y0 or NS0 CAMPATH-1H antibody by hydrazinolysis. The proportion of sialylated oligosaccharides was estimated following release of *N*-acetylneuraminic acid (NeuNAc) and quantitation by HPAEC. The low levels of NeuNAc observed suggested the presence of <5% sialylated oligosaccharides in all antibodies.

Table I. Relative retention times of N-linked oligosaccharide standards by HPAEC-PAD

Oligosaccharide structure[a]		HPAEC-PAD[b] (retention time)	[M + Na⁺] molecular ion by LD-MS
002300		0.39	934
002301		0.33	1080
013300		0.51/0.58[c]	1298
003301		0.38/0.42[c]	1283
013301		0.45/0.52[c]	1444
004300		0.69	1340
014300		0.78	1502
024300		0.91	1664
004301	1	0.59	1486
014301	2	0.68	1649
024301	3	0.78	1811
004310		0.84	1544
014310		0.94	1706
024310		1.06	1868
004311		0.74	1690
014311		0.83	1852
024311		0.94	2014

[a]Nomenclature for oligosaccharide structure in Materials and methods.
[b]Retention times relative to an internal standard of NeuNAc.
[c]Structures were derived from exoglycosidase treatment of CHO oligosaccharide Peak II (014301), as described in Table II and Scheme 1. Two peaks suggest isomers are being separated.

Oligosaccharides were separated by HPAEC (Figure 1) and showed significant differences in their profiles.

Oligosaccharides from CHO-derived CAMPATH-1H, whether grown in the absence (Figure 1A) or presence (Figure 1B) of serum, gave the simplest profile. Comparison of the relative retention times of the three main peaks (I–III; Table II) with those of standards suggested the presence of a major fucosylated series (Figure 2; Family 1) whose members differed in terminal galactosylation. LD-MS of the unfractionated oligosaccharides. (Figure 3A) confirmed the presence of this series, as well as a minor non-fucosylated series (Figure 2, Family 2). Each of the main peaks (I–III) was separated by chromatography on HPAEC and further analysed by LD-MS. LD-MS of Peak I (Figure 3B) indicated a [M + Na⁺] molecular ion at 1487, suggesting the non-galactosylated structure, 004301; Peak II (Figure3C) had a [M + Na⁺] molecular ion at 1645, indicating the monogalactosylated structure, 014301; lastly, Peak III (Figure 3D) was a mixture of two major [M + Na⁺] molecular ions at 1501 and 1809, indicative of the monogalactosylated, non-fucosylated structure, 014300, and the digalactosylated structure, 024301, respectively. Each of the fractionated oligosaccharides (Peaks I–III), as well as the unfractionated oligosaccharides, was treated with exoglycosidases and analysed by HPAEC and LD-MS. The strategy of exoglycosidase digestion is shown in Scheme 1 and the results, summarized in Table II. are fully consistent.

Oligosaccharides from Y0-derived CAMPATH-1H gave a far more complicated profile (Figure 1C), although the major peaks (I–III) that dominated the CHO oligosaccharide profile were present. In addition, however, the presence of a fucosylated and non-fucosylated series containing a bisecting GlcNAc (Figure 2: Family 3 and Family 4, respectively) was indicated, based on the relative retention times of standard oligosaccharides. LD-MS of the unfractionated oligosaccharides was fully consistent with this interpretation (Table III). Confirmation of the assigned structures was obtained by exoglycosidase

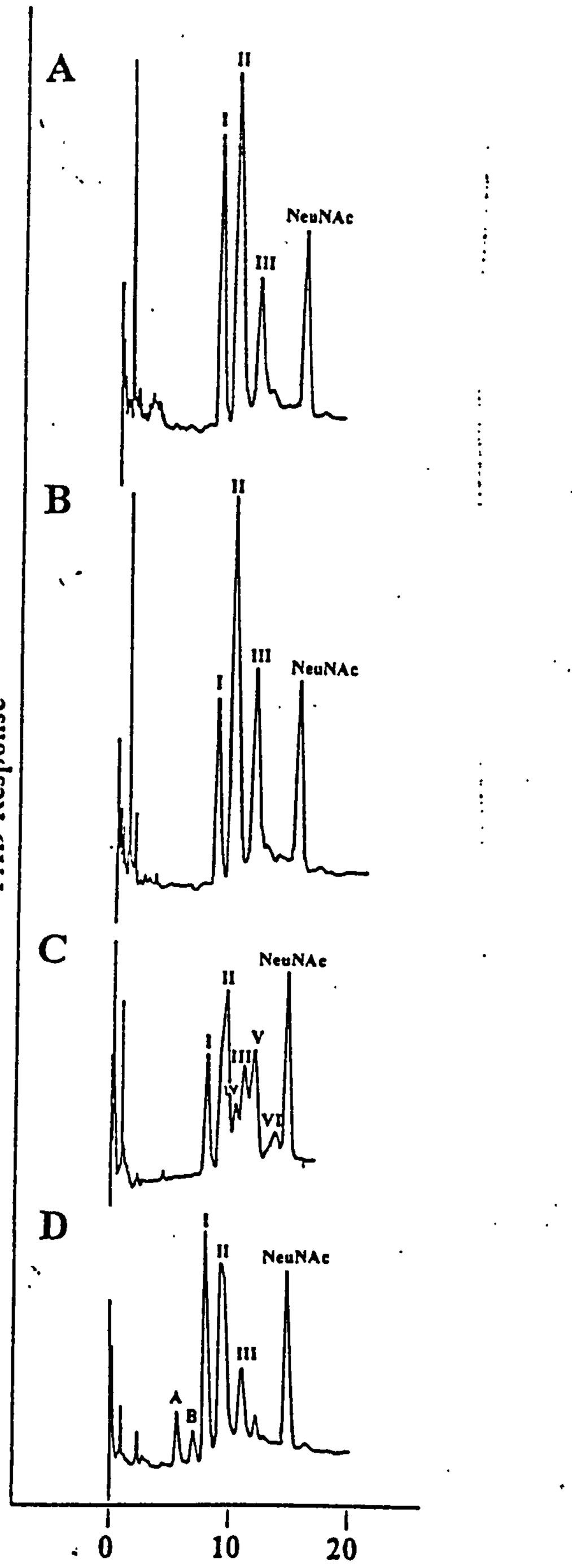


Fig. 1. HPAEC-PAD of oligosaccharides released by hydrazinolysis treatment of CAMPATH-1H. (A) CHO (serum-free culture conditions); (B) CHO (serum-containing culture conditions); (C) Y0; (D) NS0. An internal standard of NeuNAc was included.

Table II. Structural determination of N-linked oligosaccharides from CHO-derived CAMPATH-1H

Oligosaccharide[a]	Exoglycosidase treatment[b]	HPAEC[c] (peak no.)	MW (LD-MS)[d]	Structure[e]
Unfraction-ated	None	0.59 (I)	1484	1a (004301)
		0.67 (II)	1646	1b (014301)
			1338*	2a (004300)
		0.78 (III)	1809*	1c (024301)
			1501*	2b (014300)
	G	0.58	1486	1a (004301)
		0.68*	1340	2a (004300)
	F	0.68	1339	2a (004300)
		0.77	1501	2b (014300)
		0.89	1664	2c (024300)
	G+F	0.68	1339	2a (004300)
Peak I	None	0.59	1487	1a (004301)
	H	0.32	1079	002301
	H+F	0.38	934	002300
Peak II	None	0.68	1645	1b (014301)
	G	0.57	1483	1a (004301)
	H	0.45/0.52[f]	1444	013301
	H+G	0.38/0.42[f]	1283	003301
	H+F	0.51/0.58[f]	1298	013300
Peak III	None	0.78	1809	1c (024301)
			1501	2b (014300)
	G	0.58	1485	1a (004301)
		0.67	1340	2a (004300)
	F	0.90	1664	2c (024300)
		0.78	1501	2b (014300)

*Minor peak.
[a]Released from CAMPATH-1H by hydrazinolysis and fractionated on HPAEC (see Figure 1A and B).
[b]Oligosaccharides were incubated with exoglycosidases prior to analysis. G = β-galactosidase; F = α-fucosidase; H = β-*N*-acetylhexosaminidase; + indicates treatment with a mixture of exoglycosidases.
[c]Retention time relative to NeuNAc (see Table I).
[d]Molecular weight of the [M + Na⁺] ion (see Table I).
[e]Deduced structure (see Table I and Figure 2 for nomenclature).
[f]Two peaks observed by HPAEC, consistent with isomeric structures.

treatment of the unfractionated oligosaccharides with α-fucosidase (Figure 4A) or β-galactosidase (Figure 4B), or a combination of both α-fucosidase and β-galactosidase (Figure 4C), followed by chromatography on HPAEC and LD-MS. Treatment with α-fucosidase revealed the presence of both non-fucosylated series (Figure 2; Family 2 and Family 4), whereas treatment with β-galactosidase showed the presence of the non-galactosylated oligosaccharide from each of Families 1–4. Treatment with both exoglycosidases simplified the HPAEC trace further (Figure 4C) to the non-galactosylated oligosaccharide from each of Family 2 and 4. LD-MS of each of the exoglycosidase-treated oligosaccharide mixtures confirmed the proposed assignments. The strategy of exoglycosidase digests is shown in Scheme 2, and the results are summarized in Table III.

The profile of oligosaccharides from NS0 antibody (Figure 1D) was similar to that from CHO material. The major peaks (I–III) were again present, and their structures were deduced by LD-MS, suggesting that the major fucosylated series, Family 1, is a major contributor and the non-fucosylated series, Family 2 (Figure 2), is a minor contributor to the HPAEC profile. These structures were confirmed by exoglycosidase treatment, as described for Y0 oligosaccharides, followed by HPAEC and LD-MS. However, two minor peaks (Figure 1D; Peak A and B)

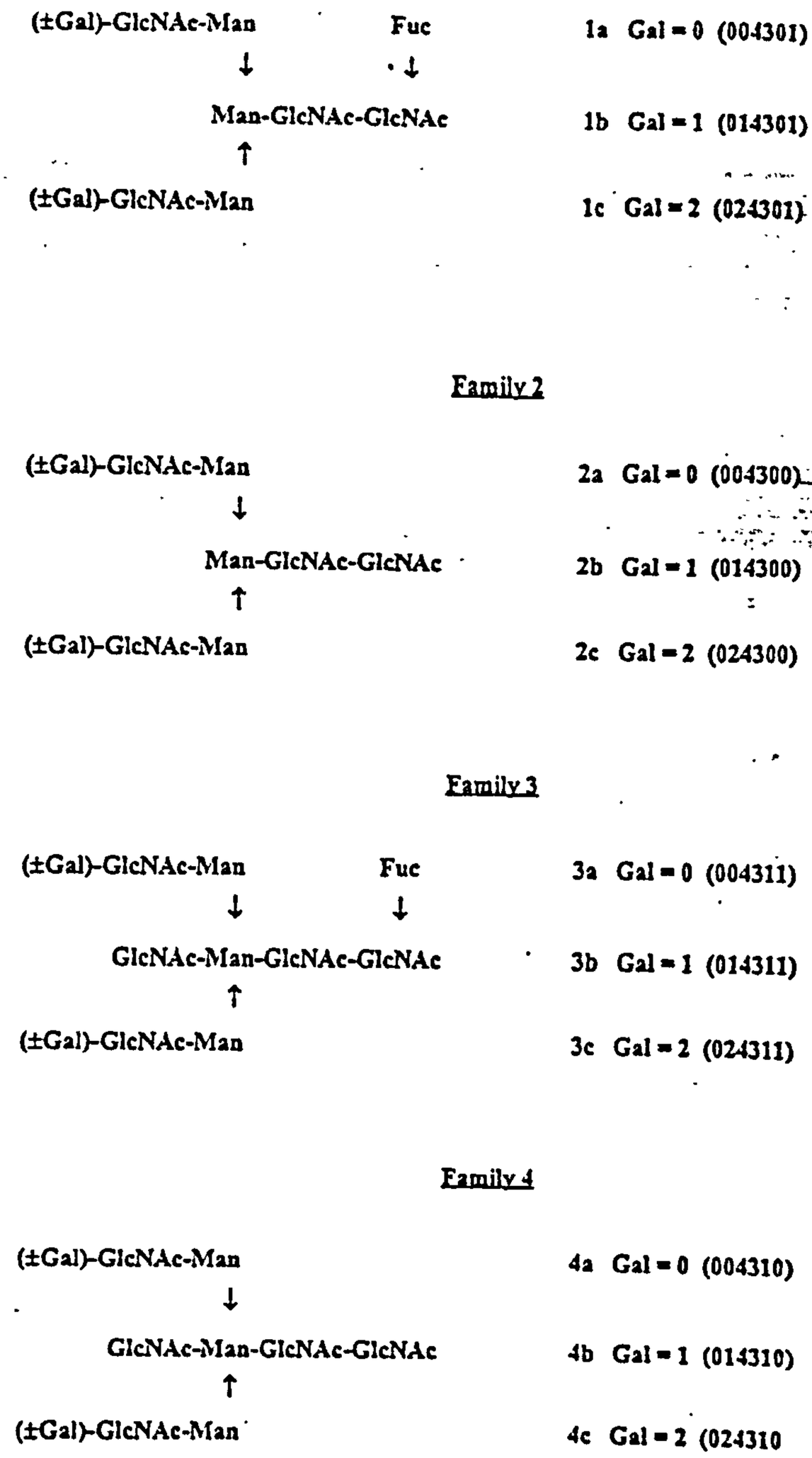


Fig. 2. Typical complex N-linked oligosaccharide structures found on the CH_2 domain of the Fc region of IgG antibody. Terminal NeuNAc is omitted for simplicity. Shorthand nomenclature is in parentheses.

with short retention times were observed, that were not found in the CHO or Y0 oligosaccharide HPAEC profiles. Although these structures were not analysed in detail, they appear to be the result of underglycosylation, as outlined below.

Peak II from CHO oligosaccharides was determined to be 014301. Exoglycosidase treatment of this fraction (see Scheme 1) with β-*N*-acetylhexosaminidase (to give 013301), followed by β-galactosidase (to give 003301) or α-fucosidase (to give 013300), produced a series of underglycosylated oligosaccharides whose structures were fully consistent with LD-MS data. They could all be separated into two isomers by HPAEC, with overlapping relative retention times in the range 0.38–0.58 (Table I). The two minor peaks from NS0

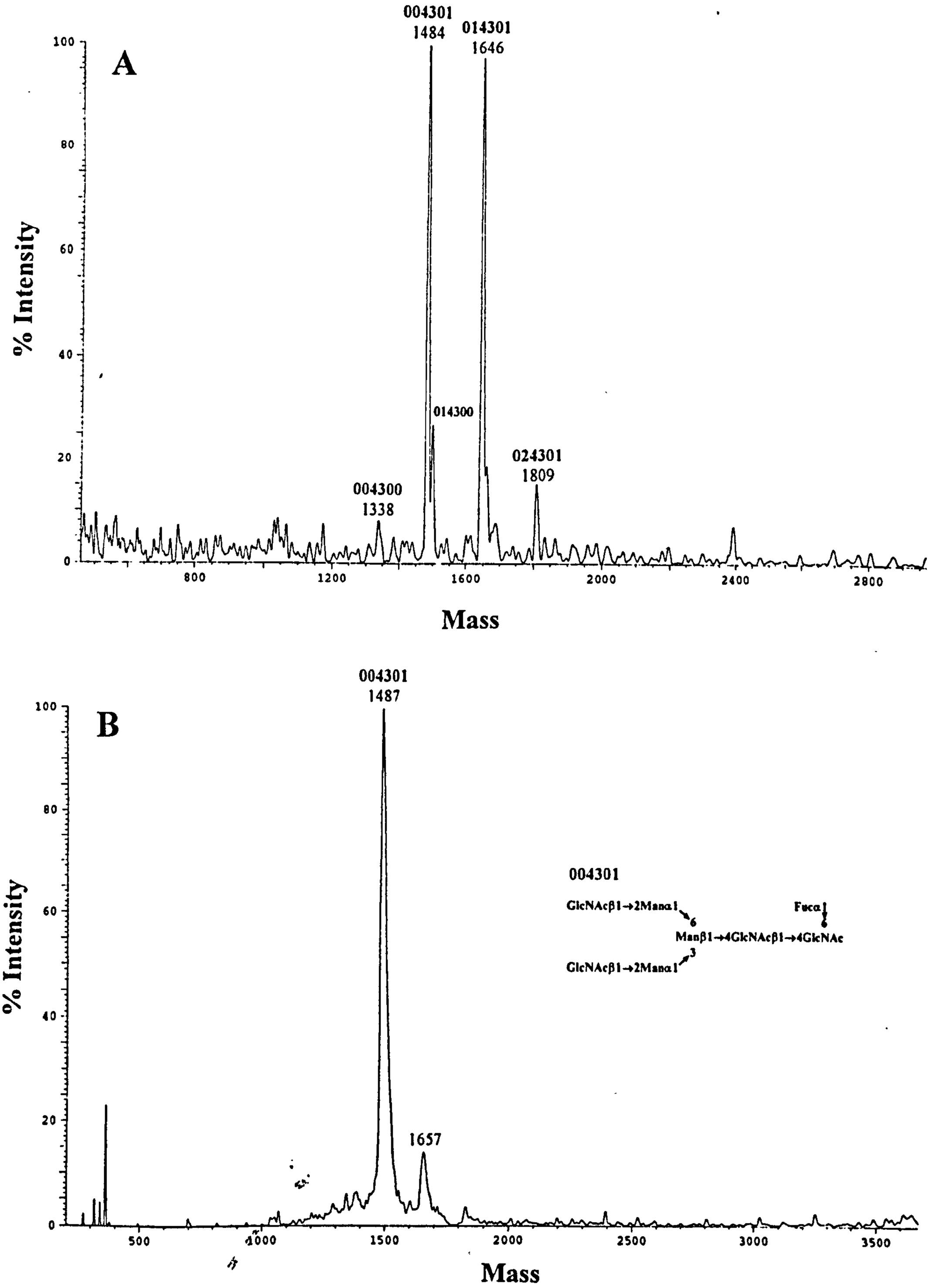
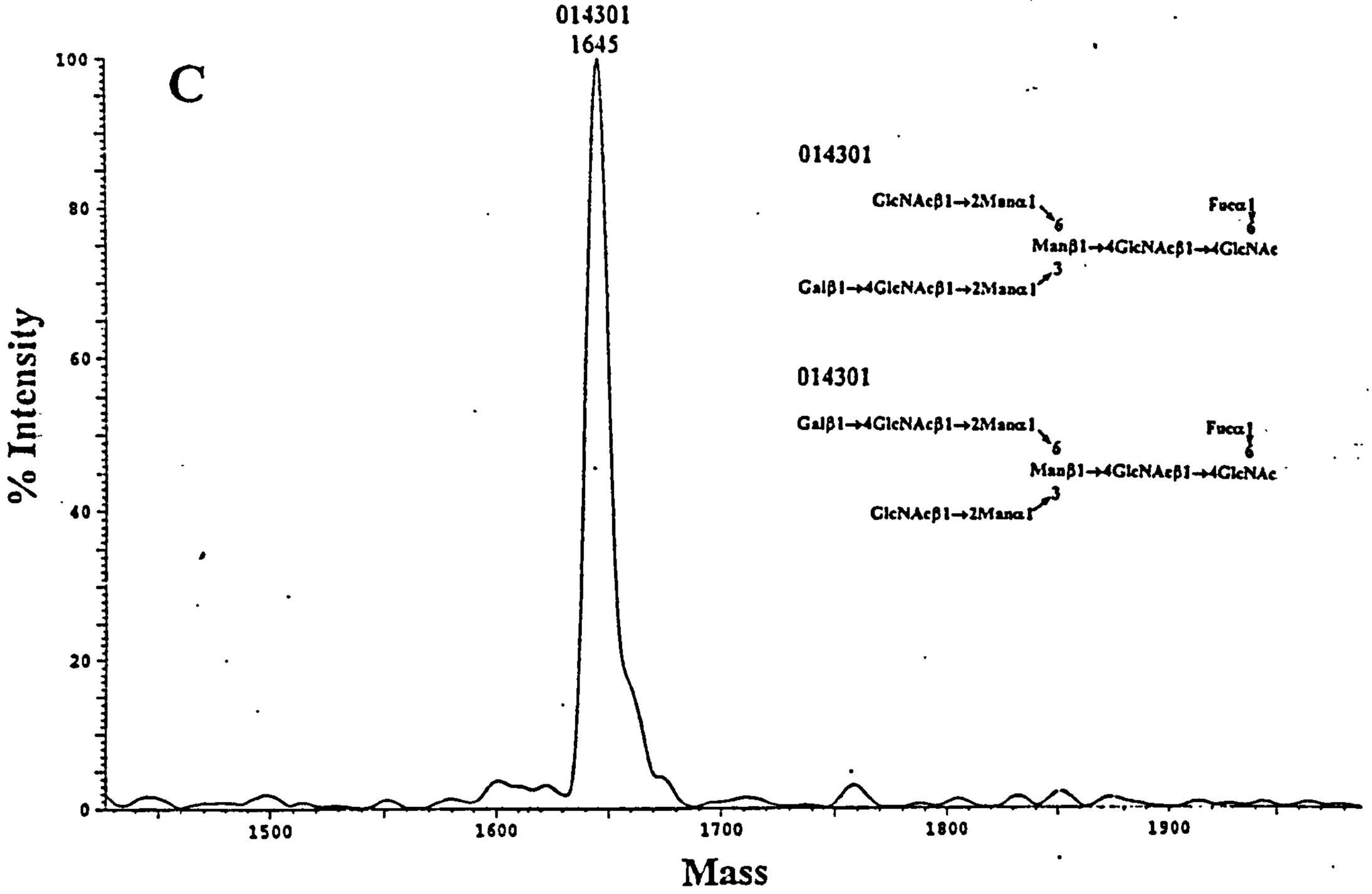
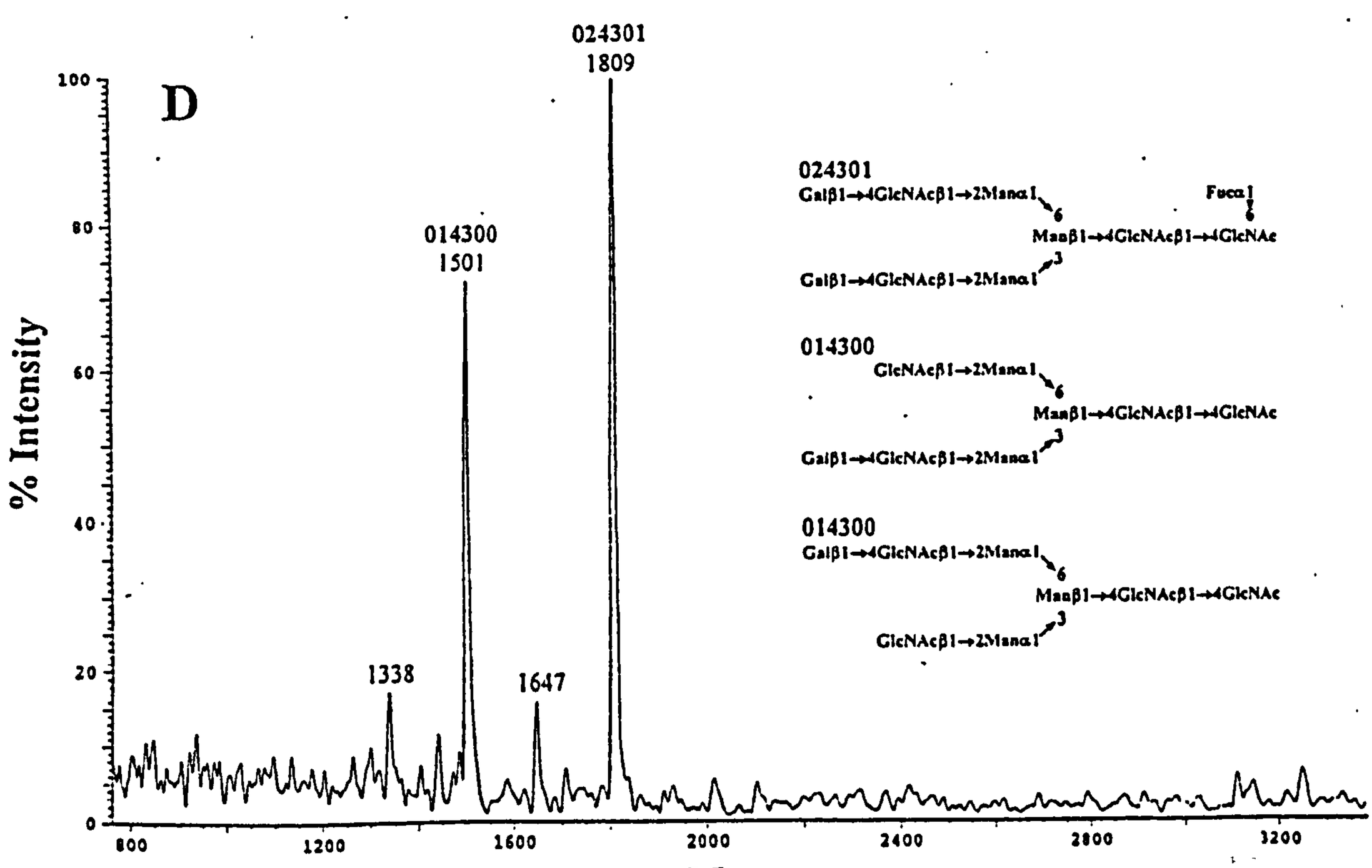


Fig. 3. LD-MS profile of oligosaccharides released by hydrazinolysis of CHO CAMPATH-1H and fractionated by HPAEC-PAD (see Figure 1A). (A) Unfractionated oligosaccharides; (B) Peak I; (C) Peak II; (D) Peak III. Assigned structures are based on retention times by HPAEC-PAD, LD-MS and exoglycosidase treatment of the oligosaccharide fractions. (Figures C and D are shown on following page.)


C
014301
1645
% Intensity
Mass
014301
GlcNAcβ1→2Manα1
6
Fucα1
6
Manβ1→4GlcNAcβ1→4GlcNAc
3
Galβ1→4GlcNAcβ1→2Manα1
014301
Galβ1→4GlcNAcβ1→2Manα1
6
Fucα1
6
Manβ1→4GlcNAcβ1→4GlcNAc
3
GlcNAcβ1→2Manα1


D
024301
1809
014300
1501
1338
1647
% Intensity
Mass
024301
Galβ1→4GlcNAcβ1→2Manα1
6
Fucα1
6
Manβ1→4GlcNAcβ1→4GlcNAc
3
Galβ1→4GlcNAcβ1→2Manα1
014300
GlcNAcβ1→2Manα1
6
Manβ1→4GlcNAcβ1→4GlcNAc
3
Galβ1→4GlcNAcβ1→2Manα1
014300
Galβ1→4GlcNAcβ1→2Manα1
6
Manβ1→4GlcNAcβ1→4GlcNAc
3
GlcNAcβ1→2Manα1

Scheme 1. Strategy for the structural determination of N-linked oligosaccharides from CHO-derived CAMPATH-1H

Oligosaccharide[a]	Sequence of exoglycosidase digests[b]
Peak I	004301 —H→ 002301 —F→ 002300
	1487[c] 1079 934
Peak II	014301 —H→ 013301 —F→ 013300
	1646 1444 1298
	G G
	↓ ↓
	004301 003301
	1483 1283
Peak III	024301 + 014300 —G→ 004301 + 004300
	1809 1501 1485 1340
	F
	↓
	024300 + 014300
	1664 1501

[a]Peaks isolated by fractionation on HPAEC (see Figure 1A and Table II).
[b]Exoglycosidase treatments: G = β-galactosidase; F = α-fucosidase; H = β-*N*-acetylhexosaminidase.
[c]Molecular weight of the [M + Na⁺] ion by LD-MS.

oligosaccharides had retention times (Figure 1D; Peak A = 0.44, Peak B = 0.52) consistent with any or all of these underglycosylated oligosaccharides. However, LD-MS of the unfractionated oligosaccharides indicated [M + Na⁺] molecular ions at 1283 and 1445, suggesting the presence of 003301 and 013301, respectively. Interestingly, oligosaccharides from an NS0-derived anti-CD4 antibody had a very similar profile, with the same evidence of underglycosylation (data not shown).

Biological activity of CAMPATH-1H

The concentration of CAMPATH-1H was measured by adsorption at OD 280 nm and by human IgG enzyme-linked immunosorbent assay (ELISA). The ability to bind to CD52 antigen by ELISA and by fluorescent activated cell scan (FACS) staining of antigen-bearing cells was similar for all antibody preparations. Surface plasmon resonance (SPR) analysis indicated no differences in the affinities (range 1.06–1.57 × 10^8/M) of the three CAMPATH-1H antibodies (Y0, NS0 and CHO) for the antigen over a range of dilutions. It can be concluded that differences in glycosylation of the various antibody preparations are not reflected in differences in the ability to bind to the CD52 antigen or in the affinity for such an interaction.

The ADCC activity of CAMPATH-1H antibodies from different cell cultures (CHO, Y0 and NS0) was measured using effector cells from a range of donors. The results shown (Figure 5) are typical of several assays, using isolated donors on each occasion, and indicate that, at the lower doses utilized in the assay, Y0 antibody was consistently more active. These results are exemplified by the concentration required to achieve 50% specific lysis (i.e. 0.0001 μg/ml for the Y0 antibody, and 0.001 and 0.001–0.01 μg/ml for the NS0 and CHO antibodies, respectively). As expected, removal of N-linked oligosaccharides from CAMPATH-1H abrogated activity (data not shown).

In contrast, no differences between the antibodies in the monocyte killing assay were found, although good activity was observed in all cases (data not shown).

Table III. Structural determination of N-linked oligosaccharides from Y0-derived CAMPATH-1H

Oligosaccharide[a]	Exoglycosidase treatment[b]	HPAEC[c] (peak no.)	MW (LD-MS)[d]	Structure[e]
Unfractionated	None	0.59 (I)	1486	1a (004301)
		0.69 (II)	1647	1b (014301)
			1340	2a (004300)
		0.74 (IV)	1688	3a (004311)
		0.78 (III)	1808	1c (024301)
			1501	2b (014300)
		0.83 (V)	1850	3b (014311)
			1543	4a (004310)
		0.94 (VI)	1704	4b (014310)
	G	0.59	1484	1a (004301)
		0.69	1340	2a (004300)
		0.73	1686	3a (004311)
		0.84	1545	4a (004310)
	F	0.69	1340	2a (004300)
		0.78	1502	2b (014300)
		0.84	1542	4a (004310)
		0.91	1664	2c (024300)
		0.93	1704	4b (014310)
	G+F	0.69	1339	2a (004300)
		0.83	1544	4a (004310)

[a]Released from CAMPATH-1H by hydrazinolysis and fractionated on HPAEC (see Figure 1C).
[b]Oligosaccharides were incubated with exoglycosidases prior to analysis. G = β-galactosidase; F = α-fucosidase; H = β-*N*-acetylhexosaminidase; + indicates treatment with a mixture of exoglycosidases.
[c]Retention time relative to NeuNAc (see Table I).
[d]Molecular weight of the [M + Na⁺] ion (see Table I).
[e]Deduced structure (see Table I and Figure 2 for nomenclature).

Discussion

It is known that both the culture conditions and the cell line can affect the glycosylation pattern of an antibody. It is equally clear that glycosylation plays a role in the biological activity of the antibody. This study was initiated to determine whether expression of the clinically important, humanized monoclonal IgG1 antibody, CAMPATH-1H, from different cell lines had an effect on glycosylation and, if so, whether *in vitro* biological activity was also altered.

The results from this study demonstrate that major differences in antibody glycosylation occur between cell lines, and minor differences occur for a given cell line grown under different culture conditions. Glycosylation patterns differ markedly between the antibodies expressed in CHO (Figure 1A), Y0 (Figure 1C) and NS0 (Figure 1D) cells. Y0 CAMPATH-1H is remarkable in being the only antibody with a bisecting GlcNAc on the core oligosaccharide (see Figure 2; Family 3 and 4), and there is evidence to suggest that the NS0 antibody has a significant degree of underglycosylation (Figure 1D; Peaks A/B). In contrast, the different culture conditions used in this study result in less significant effects on CHO antibody glycosylation (Figure 1A and B), although quantitative differences are observed, suggesting that a higher proportion of oligosaccharide chains have terminal galactose residues when grown in serum-containing medium (Figure1B; Peak III > Peak I) rather than protein-free medium (Figure1A; Peak I > Peak III).

CAMPATH-1H binds to an antigen (CD52), abundantly expressed on the surface of both normal and malignant

Scheme 2. Strategy for the structural determination of N-linked oligosaccharides from Y0-derived CAMPATH-1H

Sequence of exoglycosidase digests[a]		
004301 (1486)[b] 014301 (1647) 024301 (1808)		004301 (1484)
004300 (1340) 014300 (1501)	—G→	004300 (1340)
004311 (1688) 014311 (1850)		004311 (1686)
004310 (1543) 014310 (1704)		004310 (1545)
F ↓		F ↓
004300 (1340) 014300 (1502) 024300 (1664)	—G→	004300 (1339)
004310 (1542) 014310 (1704)		004310 (1544)

[a]Exoglycosidase treatments: G = β-galactosidase; F = α-fucosidase.
[b]Molecular weight of the [M + Na⁺] ion by LD-MS.

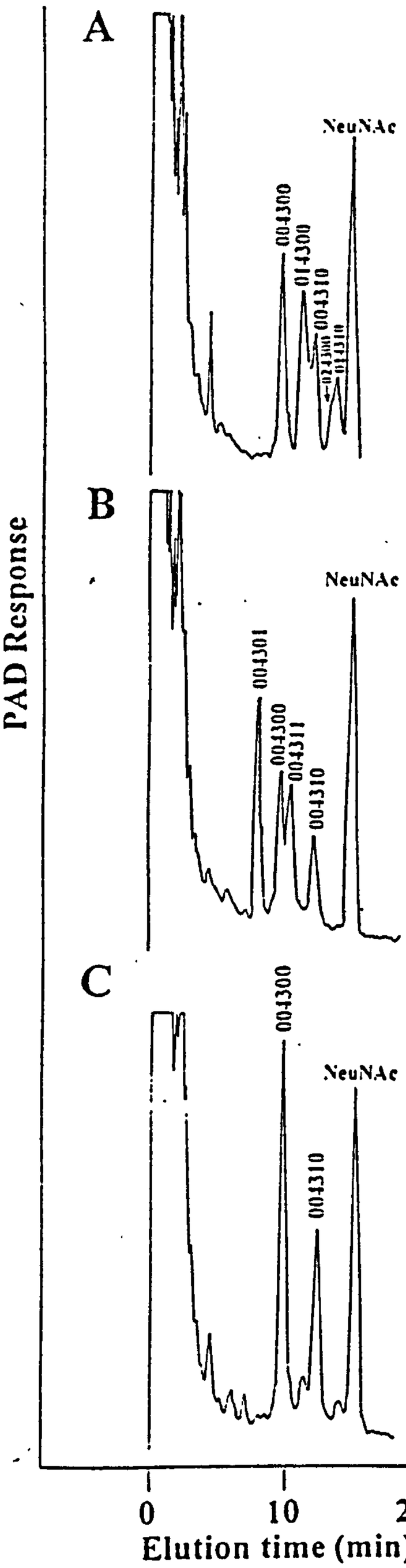


Fig. 4. HPAEC-PAD of oligosaccharides released by hydrazinolysis treatment of Y0 CAMPATH-1H, followed by exoglycosidase treatment with (A) α-fucosidase, (B) β-galactosidase, (C) α-fucosidase and β-galactosidase. An internal standard of NeuNAc was included. Shorthand nomenclature is used to assign peak structures.

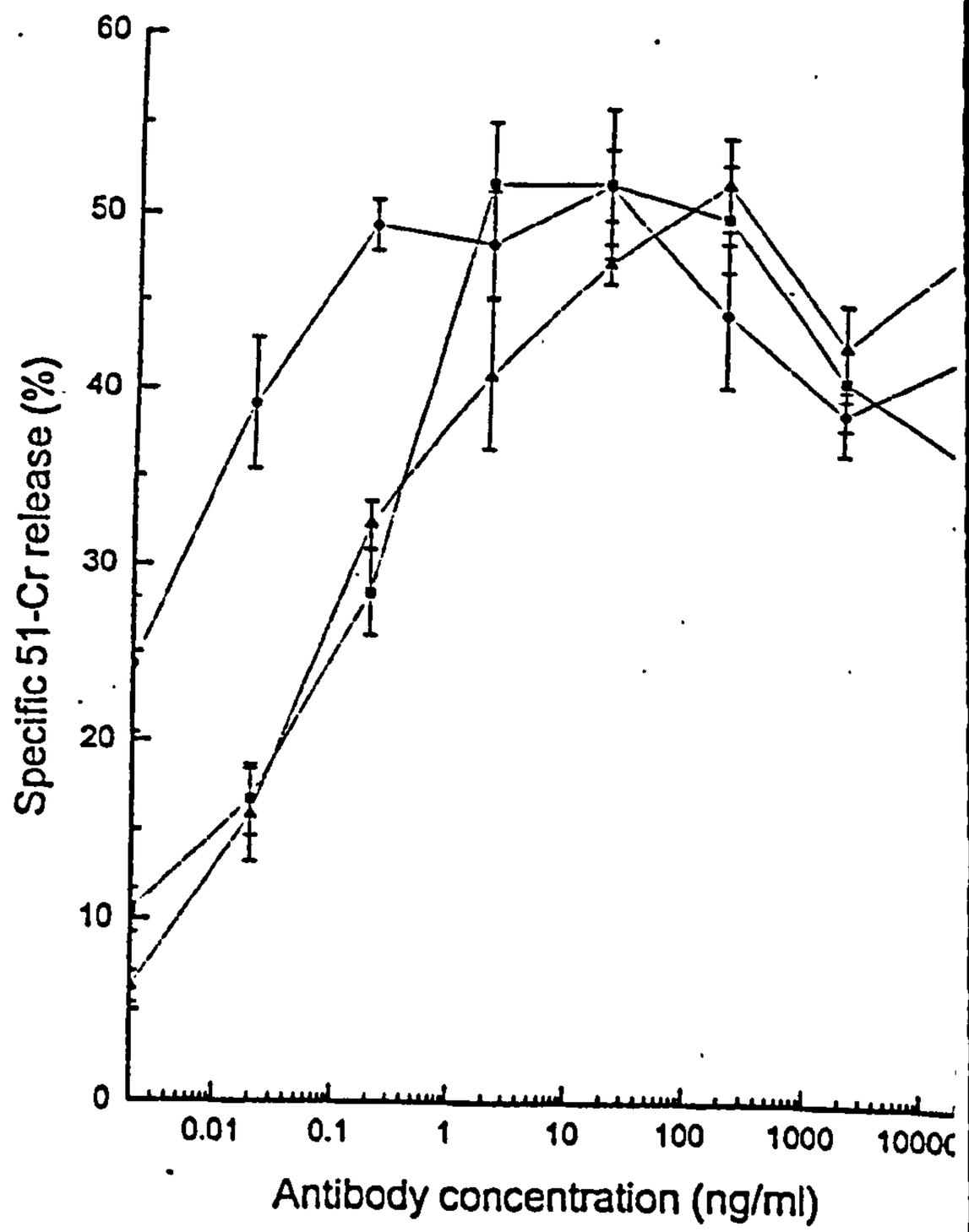


Fig. 5. ADCC activity of CHO (▲), Y0 (●) and NS0 (■) CAMPATH-1H antibodies, measured by specific release of ^{51}Cr from Wien 133, a human B-cell lymphoma cell line. SEs of triplicate samples are included.

lymphocytes, and is remarkably effective in both CML and ADCC *in vitro*. The antibody has been in major clinical trials for several years, and has proved to be very effective in lymphocyte depletion. The mechanism of lymphocyte depletion does not appear to be via CML, since CAMPATH-1M, a rat IgM, has excellent CML activity *in vitro* but is ineffective *in vivo*. In contrast, there is a good correlation between ADCC activity *in vitro* and lymphocyte depletion in patients.

In this study, the biological activity of CAMPATH-1H was measured by both ADCC and monocyte killing. The ADCC assay measures the capacity of $CD16^+$, $CD56^+$ killer cells (effector cells) to lyse antibody-coated target cells. The specific lytic mechanism is via the CD16 receptor on the effector cell, which binds to the Fc CH_2 region of IgG when bound to the target cell, and can be affected by the glycosylation pattern of the antibody. The monocyte killing assay is performed over a 5 day time period and utilizes cells of the $CD14^+$ lineage. The mechanism of action is also mediated via antibody interactions, but is monitored by the growth inhibition of the target cells rather than by killing. The IgG Fc receptors found displayed on the monocytes vary with time in culture, and a combination of CD16, CD32 and CD64 is thought to be involved.

Although all antibodies had similar activity in the monocyte killing assay, Y0 CAMPATH-1H had enhanced activity over both NS0 and CHO antibodies in the ADCC assay. Moreover, the deglycosylated antibody had no ADCC activity, demonstrating the requirement for an N-linked oligosaccharide on the CH_2 region of the heavy chain. This result suggests a relationship between the glycosylation pattern and biological activity, and that oligosaccharide structures containing a bisecting GlcNAc may play an important role. The implication for clinical efficacy is obvious: should the mechanism of lymphocyte depletion *in vivo* be via ADCC rather than monocyte killing activity, then Y0 CAMPATH-1H would have a significant benefit over CHO or NS0 antibody. It is interesting that, in this study, antibody glycosylation has a markedly different effect on the ADCC and monocyte killing activities, even though the mechanisms of action are both mediated via Fc receptor binding to antibody. It would not be safe to extrapolate these results to other antibodies.

One means of gaining a better understanding of the role of antibody glycosylation in biological systems would be to remove monosaccharide residues from the oligosaccharide chain on an intact antibody molecule in a stepwise fashion. Preliminary experiments in our laboratory demonstrated the feasibility of this approach with CAMPATH-1H (M.R.Lifely, unpublished observations), in which successive treatments with the exoglycosidases, *N*-acetylneuraminidase, β-galactosidase and *N*-acetyl-β-hexosaminidase, removed the appropriate residues, albeit with increased difficulty, without affecting binding activity.

Materials and methods

Source of CAMPATH-1H

The CHO 3D11 cell line expressing CAMPATH-1H using dihydrofolate reductase as a selectable marker (Page and Sydenham, 1991) was grown in either a hollow fibre fermenter (Acusyst-Jr) culture using Iscove's medium lacking HEPES supplemented with non-essential amino acids, 1 μM methotrexate and 5% fetal bovine serum (FBS), or using Wellcome culture medium 5 (WCM5) serum-free medium with 1 μM methotrexate in a stirred 8000 l bioreactor. WCM5 contains human recombinant insulin, but is otherwise protein free.

Y0 antibody was prepared using transfectant TF57 (Riechmann *et al.*, 1988). The cells were grown in roller bottles using Iscove's medium supplemented with 2% FBS and hypoxanthine/aminopterin/thymidine.

For re-expression in NS0 cells, the heavy and light chain CAMPATH-1H cDNA was recloned into glutamine synthetase selection-based vectors (Bebbington *et al.*, 1992) obtained from Celltech, Slough, UK, transfected by electroporation, then amplified with 5 μM methionine sulphoximine. NS0 9D4, a clonal cell line, selected in serum-containing medium, was transferred to protein-free growth in WNSD medium, which is of defined composition being free of protein and other large-molecular-weight components. After several weeks of protein-free growth, in shake flasks, culture supernatant was harvested.

Antibody purification

The culture supernatant containing CAMPATH-1H from NS0 9D4 cells was clarified by centrifugation and passed through a 0.2 μm filter. The antibody bound to a protein A–Sepharose column was eluted with 0.1 M citrate buffer (pH 3), bound to a S-Sepharose ion-exchange column, then eluted with 20 mM citrate–phosphate buffer containing 200 mM NaCl. A stock solution at 5.54 mg/l was prepared and stored at 4°C. Clinical Trial Material grade, serum-free CHO 3D11-derived CAMPATH-1H was obtained as a 10 mg/ml solution in phosphate-buffered saline (PBS) in a sealed glass ampoule, and stored at 4°C. Clarified supernatants from CHO 3D11 or Y0 TF57 were purified using a similar protocol to that above. The S-Sepharose eluate was concentrated, desalted, and separated from dimer and higher aggregates using a Superdex 200 size-exclusion column and stored in PBS.

Release of oligosaccharides by hydrazinolysis

CAMPATH-1H (5 mg) was dialysed against water to remove salts and freeze–dried in a glass ampoule. Hydrazine (0.1 ml) was added and the sealed ampoule heated at 95°C for 5 h. The released oligosaccharides were separated from hydrazine and peptides by adsorption to a cellulose (Whatman, CF-11) column (Bio-Rad; 1 ml) in butanol:ethanol:acetic acid (4:1:0.5 v/v/v; 1.5 ml). After washing the cellulose in butanol:ethanol:water (4:1:1 v/v/v; 3 × 1 ml), oligosaccharides were *N*-reacetylated *in situ* with acetic anhydride:methanol (2:5 v/v; 1.4 ml) for 30 min at room temperature. The column was washed with butanol:ethanol:water (4:1:1 v/v/v; 4 × 1 ml), then with methanol (1 ml). Oligosaccharides were then eluted from the column with 0.2 M sodium acetate (2 × 1 ml), further treated with acetic anhydride (0.1 ml) for 30 min then with 0.1 M copper acetate (0.1 ml) for 30 min to convert the acetylhydrazone derivatives to the unreduced oligosaccharides. Cations (Na^+ and Cu^+) were removed on an ion-exchange stack column of Chelex 100 (H^+ form; upper; 0.4 ml) and AG-50 (H^+ form; lower; 1 ml), and oligosaccharides were recovered as the aqueous eluant, before lyophilization.

Desialylation of oligosaccharides

Oligosaccharides were treated with 40 mM trifluoroacetic acid at 80°C for 1 h under helium to remove sialic acid. Trifluoroacetic acid was removed under a stream of nitrogen at 40°C, and sialic acid content was measured by HPAEC using the conditions for separation of oligosaccharides described above.

Exoglycosidase treatment

The following exoglycosidases were obtained from Oxford Glycosystems: G, β-galactosidase from bovine testes (x-5013); F, α-fucosidase from bovine epididymis (X-5006); H, β-*N*-acetylhexosaminidase from jack bean meal (X-5003). Oligosaccharides (5–10 μg) were incubated with enzyme, following the manufacturer's instructions, at 37°C for 48 h in a total volume of 50 μl. Samples were purified by passage through a column of Dowex AG 50 (H^+ form; 0.3 ml) layered upon Dowex AG3 (OH^- form; 0.3 ml). The column was eluted with 3 ml water and the eluate lyophilized.

Nomenclature of N-linked oligosaccharides

N-Linked oligosaccharides present on IgG are made from a core of three mannose (Man) and two GlcNAc residues (see, for example, Figure 2). Additional GlcNAc, Gal and NeuNAc residues may be present on the two outer arms, and Fuc may be present on the penultimate GlcNAc core residue. Finally, a bisecting GlcNAc may be present, attached to the core Man. The nomenclature to describe the oligosaccharide structures uses a six-numbered digit which (from left to right) describes the number of NeuNAc (0–2), Gal (0–2), core + outer arm GlcNAc (2–4), bisecting GlcNAc (0 or 1) and Fuc (0 or 1) residues.

Analysis of oligosaccharides by HPAEC-PAD

Oligosaccharides released by hydrazinolysis were analysed by HPAEC using a Dionex Bio LC system and a Carbopak PA-1 column, eluted with a linear

gradient from 24 to 36 mM sodium acetate in 125 mM NaOH over 20 min at a flow rate of 1 ml/min, and detected by pulsed amperometric detection (PAD) using a gold electrode. An internal standard of NeuNAc was included in order to determine relative retention times of oligosaccharide peaks. Standard oligosaccharides, M3N2F (002301), NGA2F (004301), NA2F (024301), NGA2FB (004311), NA2FB (024311) and RP-2501 (a mixture of 024300, 014301, 024301, 004311, 014311, 024311) were purchased from Oxford Glycosystems. Further standards were derived from α-fucosidase treatment of 002301, 004301, 024301, 004311 and 024311, which gave 002300, 004300, 024300, 004310 and 024310, respectively. In addition, α-fucosidase treatment of RP-2501 gave a mixture of 024300, 014300, 024300, 004310, 014310 and 024310.

Preparative isolation of CHO oligosaccharide Peaks I–III

The oligosaccharide mixture (30 µl) was injected and oligosaccharides separated by HPAEC. Each peak was collected separately, desalted on AG-50 (H^+ form) and rotary evaporated to dryness to remove acetic acid. The residue was dissolved in water and a small proportion was reinjected to confirm lack of contamination with other peaks. Each fraction was lyophilized and stored at −20°C.

Structural determination of oligosaccharides by LD-MS

Samples of desialylated oligosaccharides (1–20 pmol) in water (1 µl) were mixed with the matrix component 2,3-dihydroxy benzoic acid (1 µl of a 10 mg/ml solution in 50% w/v ethanol) and an aliquot (1 µl) allowed to dry on a sample slide. Samples were ionized following irradiation with a pulse of laser light, and were analysed with a LASERMAT time-of-flight mass spectrometer (Finnigan MAT).

IgG ELISA

Culture supernatants were clarified by centrifugation at 13 000 r.p.m. in a microfuge. The wells of a 96 well maxisorp ELISA plate (Nunc, Roskilde, Denmark) were coated with sheep anti-human IgG (Seralab, UK, catalogue no. SDL2015) in PBS (2 µg/ml; 100 µl) and incubated overnight at 4°C. The excess protein binding sites were blocked by incubation with 1% bovine serum albumin (BSA) fraction V in PBS (200 µl/well) at room temperature for 1 h. The wash buffer used between each step was 0.05% Tween 20–PBS. CAMPATH-1H standard samples (2-fold dilutions from 250 ng/ml) or culture supernatants in triplicate in 0.2% BSA, 0.05% Tween 20–PBS (PBT) were added (100 µl/well) and the plate incubated at room temperature for 1.5 h. The plate was washed five times and peroxidase-conjugated sheep anti-human IgG (Sigma) was added (diluted 1:2000 in PBT;100 µl/well). The plates were incubated at room temperature for 40 min, then washed five times. Tetra-methyl benzidine as substrate (Sigma, Poole, UK) was added (100 µl/well) and incubated at room temperature until a discernible blue colour was apparent. The reaction was stopped with 2 M H_2SO_4 (100 µl/well) and the colour measured at an adsorbance of 450 nm.

CAMPATH antigen binding ELISA

This was performed as described previously (Xia *et al.*, 1993).

Antibody-dependent cell-mediated cytotoxicity assay

Peripheral blood mononuclear cells (PBMC) were separated from defibrinated fresh human blood by centrifugation over a lymphoprep (Nycomed) gradient. Wien 133, a human B cell lymphoma cell line (Nacheva *et al.*, 1987) expressing the CD52 antigen on its cell surface membrane, was grown in log phase, labelled with ^{51}Cr, washed, resuspended at 2×10^5/ml, then aliquoted into 96 well 'U'-bottomed tissue culture plates at 50 µl/well. Wien 133 cells were cultured using Iscoves's medium, 10% FBS and 2 mM L-glutamine (Wien growth medium). CAMPATH-1H antibody dilutions (50 µl), in Wien growth medium, were added to triplicate wells in the plate. Nine control wells without antibody were also included in each assay. The plates were incubated at 37°C for 1.5 h in a humid CO_2 incubator prior to the addition of 100 µl of PBMC effector cells at a concentration of 2.5×10^6/ml, in Wien growth medium, to each antibody-containing well. This resulted in a ratio of 25 effector cells for each target cell. The effector cells were also added to one triplicate set of control wells (non-antibody-mediated lysis control). Wien growth medium alone was added to yet another set of control triplicates (spontaneous lysis control) and 100 µl of 1% w/v Triton X100 were added to the final set of control wells (total release control). The plates were centrifuged for 5 min at 1500 r.p.m. before being replaced at 37°C for 5 h. At the end of the incubation period, 100 µl of medium were removed from each well and counted in a γ counter (Wallac Wizard, Model 1470, Turku, Finland). The level of specific release was calculated as follows:

$$\% \text{ specific release} = \frac{(\text{mean c.p.m. antibody-mediated release}) - (\text{mean c.p.m. spontaneous release})}{(\text{mean c.p.m. total release}) - (\text{mean c.p.m. spontaneous release})} \times 100$$

Monocyte killing assay

Human peripheral blood monocytes were isolated from PBMC by adherence to plastic. This was performed by diluting PBMC in RPMI 1640 medium containing 10% autologous serum (RPMI-S) and incubating for 2 h at 37°C in a 75 cm^2 tissue culture flask. Non-adherent cells were removed by washing twice with RPMI 1640 medium. Monocytes were subsequently cultured for ~14 days in RPMI-S and then assayed for CD14 expression by FACS. The cells were harvested and dispensed into 96 well plates at 10^5/ml and 100 µl/well together with 4×10^3 CD52-expressing Wien 133 target cells. A range of CAMPATH-1H antibody dilutions, in triplicate, was then added and the plates incubated for 5 days. Target cell proliferation rate was assessed by incorporation of [^{3}H]thymidine. Growth inhibition due to monocyte cytotoxicity was expressed as percentage inhibition in comparison with duplicate cultures in the absence of monocytes.

Fluorescent activated cell scan

To compare antigen binding of the different antibodies, Wien 133 cells were washed and resuspended in PBS containing 5% FBS, 0.1% sodium azide at 10^5/ml, and 100 µl aliquoted into the wells of a 96 well plate. A range of antibody dilutions were subsequently added to the wells and the plate placed at 4°C for 30 min. After washing in the same buffer, the cells were resuspended in 100 µl of fluorescein isothiocyanate-labelled anti-human IgG diluted 1 in 100 and again placed at 4°C for 20 min. After extensive washing, the cells were resuspended in PBS containing 1% v/v paraformaldehyde and analysed on a Becton Dickinson FACscan.

Surface plasmon resonance binding

Real-time biospecific interaction analysis using a BIAcore™ (Pharmacia Biosensor AB, Sweden) with SPR was used to determine rate and affinity constants for the various preparations of CAMPATH-1H. This was accomplished by activating the surface of a dextran-coated gold chip with the amine coupling kit (Pharmacia) and coupling the purified CAMPATH antigen, CD52 (Xia *et al.*, 1993) in 10 mM HEPES, 1 M NaCl, pH 7.4 (0.4 nmol CD52 in 80 µl). CAMPATH-1H, diluted to 3–100 µg/ml in 10 mM HEPES, 150 mM NaCl (pH 7.4), 0.05% BIAcore Surfactant P20 (HBS), was exposed to the coupled antigen for 6 min at a flow rate of 5 µl/min. This was followed by a dissociation phase in which HBS alone was injected. Finally, the antigen surface was regenerated by exposure to 10 mM NaOH for 1 min and monitoring binding of the antibody to the antigen.

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Abbreviations

ADCC, antibody-dependent cellular cytotoxicity; BSA, bovine serum albumin; CHO, Chinese hamster ovary; CML, complement-mediated lysis; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescent activated cell scan; FBS, fetal bovine serum; Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylglucosamine; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; LD-MS, laser desorption-mass spectrometry; Man, mannose; NeuAc, *N*-acetylneuraminic acid; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PBT, 0.05% Tween 20–PBS; SPR, surface plasmon resonance.

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